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(54) Novel human TNF polypeptide mutants and DNAs encoding said mutants.

(57) A human TNF polypeptide mutant having an amino acid sequence of modified human TNF polypeptide, a DNA having a base sequence encoding the above human TNF polypeptide mutant and a method of producing the above human TNF polypeptide mutant by culturing a host transformed with a vector having inserted therein the above DNA. The above human TNF polypeptide mutant is soluble and has antitumor activity.

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This invention relates to novel human tumor necrosis factor (hereinafter referred to as TNF) polypeptide mutants, a process for production thereof, and DNAs encoding these mutants

TNF is a physiologically active substance discovered by Carswell et al. in 1975 [Proc. Natl. Acad. Sci., USA, 72, 3666 (1975)]. It is characterized by showing strong cytotoxic activity against tumor cells in vitro and necrotizing a transplanted tumor in vivo [L. J. Old, Cancer Res., 41, 361 (1981)].

In 1984 to 1985, DNAs encoding rabbit, human and mouse TNFs were isolated [European Patent Publication No. 146026, European Patent Publication No. 155549, European Patent Publication No. 158286 and Fransen et al., Nucleic Acids Res., 13, 4417 (1985)], and the entire primary structures of their TNF polypeptides were elucidated.

Isolation of DNAs encoding TNFs, particularly DNA encoding human TNF, enabled human TNF to be produced in microorganisms by genetic engineering techniques, and various properties of human TNF were studied in greater detail. These studies led to the determination that human TNF has strong cytotoxic activity in vitro and antitumor activity in vivo [D. Pennica et al., Nature, 312, 724 (1984); T. Shirai et al., Nature, 313, 803 (1985); and M. Yamada et al., J. Biotechnology, 3, 141 (1985)].

Studies on the mutation of human TNF polypeptides have also been undertaken, and several patents have been published (PCT International Patent Publication No. WO86/02381, PCT International Patent Publication No. WO86/04606, European Patent Publication No. 168214, European Patent Publication No. 155549 which corresponds to U. S. Patent Application Serial No. 708846, and Japanese Patent Publication No. 48632/1987). The first three patents merely refer to the mutation of human TNF

polypeptide composed of 157 amino acids or give a disclosure of specific mutations. The remaining two patents disclose or refer to the mutation of human TNF polypeptide composed of 155 amino acids. The human TNF polypeptide mutants of the present invention, however, differ in amino acid sequence from the mutants specifically disclosed in these patents.

The present inventors actually mutated amino acid(s) in the amino acid sequence of human TNF polypeptide composed of 155 amino acid residues and polypeptides resulting from deletion of amino acid(s) beginning with the N-terminus of the human TNF polypeptide, and examined the properties of the resulting human TNF polypeptide mutants. This work has led to the discovery that soluble polypeptides can be obtained only when specific amino acid(s) at specific site(s) in the above polypeptides are mutated. It is an object of this invention, therefore, to provide a group of soluble human TNF polypeptide mutants.

Another object of this invention is to provide human TNF polypeptide mutants which are soluble and have TNF activity.

The present inventors have found that certain mutants in the above group have cytotoxic activity and antitumor activity <u>in vitro</u> and <u>in vivo</u> almost comparable to those of human TNF. It is still another object of the invention therefore to provide human TNF polypeptide mutants having excellent cytotoxic activity and antitumor activity <u>in vitro</u> and <u>in vivo</u>.

It has also been found that other specific mutants in the above group surprisingly show excellent antitumor activity <u>in vivo</u> despite their very low cytotoxic activity <u>in vitro</u>, and that in these mutants, pyrogenicity which is undesirable for use as pharmaceuticals is considerably reduced. It is a further object of this invention therefore to provide human TNF polypeptide

mutants which show very low cytotoxic activity in vitro, but excellent antitumor activity in vivo, and have reduced side-effects. The fact that these mutants show excellent activity in vivo despite their low activity in vitro indicates that structures (active centers) essential to cytotoxic activity in vitro and antitumor activity in vivo which are typical biological activities of TNF do not always exist at the same site in TNF polypeptide molecule. This also leads to the presumption that active centers of various biological activities of TNF differ from one another.

Further objects of this invention will become apparent from the following description.

To simplify the description, the following abbreviations are used in the present specification and claims.

A: adenine
C: cytosine
G: guanine
T: thymine
Ala: alanine
Arg: arginine
Asn: asparagine
Asp: aspartic acid
Cys: cysteine
Gln: glutamine
Glu: glutamic acid
Gly: glycine
His: histidine
Ile: isoleucine
Leu: leucine
Lys: lysine
Met: methionine
Phe: phenylalanine
Pro: proline
Ser: serine

Thr: threonine
Trp: tryptophan
Tyr: tyrosine
Val: valine
DNA: deoxyribonucleic acid
cDNA: complementary DNA
dATP: deoxyadenosine triphosphate
dCTP: deoxycytidine triphosphate
dGTP: deoxyguanosine triphosphate
dTTP: deoxythymidine triphosphate
kbp: kilo base pairs
bp: base pairs
SDS: sodium dodecylsulfate
MW: molecular weight:
KD: kilodaltons
SD sequence: Shine-Dalgarno sequence
Meth A sarcoma: methylcholanthrene-induced sacroma

In the present specification, the base sequence shown by a single strand is the base sequence of a sense strand, and the left end is a 5'-terminus and the right end, a 3'-terminus. In the amino acid sequence, the left end is an N-terminus, and the right end, a C-terminus.

Brief Description of the Drawings

Fig. 1 shows the restriction endonuclease mapping of cloned cDNA encoding human TNF;

Figs. 2 and 3 show the steps of constructing an expression plasmid pHNY-32 (in Example 1);

Fig. 4 shows the steps of preparing a PL-DNA fragment for construction of an expression plasmid pHPL-115 (in Example 2);

Fig. 5 shows an elution pattern by high performance liquid chromatography of peptide fragments from polypeptide TNF-115L by digestion with lysyl endopeptidase (in Example 2);

Fig. 6 shows the steps of constructing an expression plasmid pHPL-147 (in Example 3);

Fig. 7 shows the steps of constructing an expression plasmid pHPL-Ser67 (in Example 4);

Fig. 8 shows the steps of constructing an expression plasmid pHPL147S67 (in Example 5);

Fig. 9 shows the steps of constructing an expression plasmid pHTR91 (in Referential Example 1);

Fig. 10 shows the steps of constructing an expression plasmid pHT147 (in Referential Example 2); and

Fig. 11 shows the steps of constructing an expression plasmid pHTP392 (in Referential Example 3).

The present invention relates to a polypeptide having an amino acid sequence represented by formula [I] in Table 1 in which at least one of the 16th, 31st to 34th, 36th, 48th, 73rd, 82nd, 85th, 89th, 94th, 97th, 98th, 103rd, 113th, 115th, 117th, 118th, 131st, 132nd, 141st to 146th, and 153rd amino acid residues is replaced by another amino acid residue, with the proviso that when the 115th amino acid residue is replaced by another amino acid residue, the 67th amino acid residue and/or the 99th amino acid residue may be replaced by another amino acid residue; and a polypeptide resulting from deletion of one or at most 8 amino successive acid residues from the N-terminus of said polypeptide.

Table 1

Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro
Val Ala His Val Val Ala Asn Pro Gln Ala
Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
Ala Asn Ala Leu Leu Ala Asn Gly Val Glu
Leu Arg Asp Asn Gln Leu Val Val Pro Ser
Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val
Leu Phe Lys Gly Gln Gly Cys Pro Ser Thr
His Val Leu Leu Thr His Thr Ile Ser Arg
Ile Ala Val Ser Tyr Gln Thr Lys Val Asn
Leu Leu Ser Ala Ile Lys Ser Pro Cys Gln
Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys
Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly
Val Phe Gln Leu Glu Lys Gly Asp Arg Leu
Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu
Asp Phe Ala Glu Ser Gly Gln Val Tyr Phe
Gly Ile Ile Ala Leu ... [I]

More specifically, the human TNF polypeptide mutants of this invention are illustrated in the following (A), (B) and (C).

(A) The human TNF polypeptide mutants which are characterized in that at least one of the following amino acid replacements is effected in the amino acid sequence of human TNF represented by formula [I]:

Replacement of
16th Ala by Val,
31st Ala by Thr,
32nd Asn by Ala, Cys, Asp, His, Ile, Arg, Ser, Thr, Val or Tyr
34th Leu by Ile,
36th Ala by Val,
48th Val by Met,
73rd Leu by Pro,
82nd Ala by Asp,
85th Tyr by His,

89th Val by Ile,
94th Ala by Thr,
97th Ser by Asn,
98th Pro by His or Leu,
103rd Thr by Pro,
113th Tyr by Cys,
115th Pro by Leu, His, Gln, Ser, Ala, Phe, Asn, Gly, Tyr, Val, Glu, Met, Ile, Asp, Trp, Lys, Arg, or Thr,
117th Tyr by His,
118th Leu by Gln,
131st Ser by Ile,
132nd Ala by Thr,
141st Asp by Tyr,
143rd Ala by Val,
144th Glu by Lys,
145th Ser by Cys,
146th Gly by Glu, and
153rd Ile by Leu.

(B) The human TNF polypeptide mutants which are characterized in that in the amino acid sequence of human TNF of formula [I] 67th Cys and/or 99th Cys is replaced by Ser and that 115th Pro is replaced by an amino acid other than Pro.

(C) The human TNF polypeptide mutants (A) or (B) in which one or at most 8 successive amino acid residues from their N-terminus are deleted.

In the above group of mutants, examples of those mutants which show excellent cytotoxic activity in vitro and antitumor activity in vivo are shown below.

Polypeptide mutants having the amino acid sequence represented by formula [I] in which
16th Ala is replaced by Val,
36th Ala is replaced by Val,
73rd Leu is replaced by Pro,
98th Pro is replaced by His or Leu,

103rd Thr is replaced by Pro,
115th Pro is replaced by His or Gln,
131st Ser is replaced by Ile, or
143rd Ala is replaced by Val.

In the above group of mutants, examples of those mutants which have low cytotoxic activity in vitro but excellent antitumor activity in vivo are shown below.

Polypeptide mutants having the amino acid sequence represented by formula [I] in which
31st Ala is replaced by Thr,
32nd Asn is replaced by Ala, Cys, Asp, His, Ile, Arg, Ser, Thr, Val or Tyr,
115th Pro is replaced by Ser, Ala, Phe, Asn, Thr, Gly, Tyr, Val, Glu, Met, Ile, Asp, Trp, Leu or Lys, or
117th Tyr is replaced by His.

Especially preferred mutants are polypeptides having the amino acid sequence of formula [I] in which
32nd Asn is replaced by Tyr, His, Asp or Ser,
115th Pro is replaced by Leu, Ser, Asp or Gly, or
117th Tyr is replaced by His.

The present invention also relates to DNAs encoding the above polypeptide mutants of this invention. The DNAs of the invention will be described below with reference to formula (A) in Table 2.

Table 2

(5') TCA TCT TCT CGA ACC CCG AGT GAC AAG
CCT GTA GCC CAT GTT GTA GCA AAC CCT CAA
GCT GAG GGG CAG CTC CAG TGG CTG AAC CGC
CGG GCC AAT GCC CTC CTG GCC AAT GGC GTG
GAG CTG AGA GAT AAC CAG CTG GTG GTG CCA
TCA GAG GGC CTG TAC CTC ATC TAC TCC CAG
GTC CTC TTC AAG GGC CAA GGC TGC CCC TCC
ACC CAT GTG CTC CTC ACC CAC ACC ATC AGC
CGC ATC GCC GTC TCC TAC CAG ACC AAG GTC
AAC CTC CTC TCT GCC ATC AAG AGC CCC TGC
CAG AGG GAG ACC CCA GAG GGG GCT GAG GCC
AAG CCC TGG TAT GAG CCC ATC TAT CTG GGA
GGG GTC TTC CAG CTG GAG AAG GGT GAC CGA
CTC AGC GCT GAG ATC AAT CGG CCC GAC TAT
CTC GAC TTT GCC GAG TCT GGG CAG GTC TAC
TTT GGG ATC ATT GCC CTG-(3') ... [A]

Specific examples of the DNA of this invention are the following (a), (b), (c) and (d).

(a) DNAs having a base sequence represented by formula [A] in Table 2 in which
16th codon GCA for Ala is replaced by codon GTA for Val,
31st codon GCC for Ala is replaced by codon ACC for Thr,
32nd codon AAT for Asn is replaced by codon GCT for Ala,
32nd codon AAT for Asn is replaced by codon TGC for Cys,
32nd codon AAT for Asn is replaced by codon GAT for Asp,
32nd codon AAT for Asn is replaced by codon CAC for His,
32nd codon AAT for Asn is replaced by codon ATC for Ile,

32nd codon AAT for Asn is replaced by codon CGA for Arg,

32nd codon AAT for Asn is replaced by codon AGC for Ser,

32nd codon AAT for Asn is replaced by codon ACT for Thr,

32nd codon AAT for Asn is replaced by codon GTC for Val,

32nd codon AAT for Asn is replaced by codon TAT for Tyr,

34th codon CTC for Leu is replaced by codon ATC for Ile,

36th codon GCC for Ala is replaced by codon GTC for Val,

48th codon GTG for Val is replaced by codon ATG for Met,

73rd codon CTC for Leu is replaced by codon CCC for Pro,

82nd codon GCC for Ala is replaced by codon GAC for Asp,

85th codon TAC for Tyr is replaced by codon CAC for His,

89th codon GTC for Val is replaced by codon ATC for Ile,

94th codon GCC for Ala is replaced by codon ACC for Thr,

97th codon AGC for Ser is replaced by codon AAC for Asn,

98th codon CCC for Pro is replaced by codon CAC for His,

98th codon CCC for Pro is replaced by codon CTC for Leu,

103rd codon ACC for Thr is replaced by codon CCC for Pro,

113th codon TAT for Tyr is replaced by codon TGT for Cys,

115th codon CCC for Pro is replaced by codon CAC for His,

115th codon CCC for Pro is replaced by codon CAG for Gln,

115th codon CCC for Pro is replaced by codon TCC for Ser,

115th codon CCC for Pro is replaced by codon GCC for Ala,

115th codon CCC for Pro is replaced by codon TTC for Phe,

115th codon CCC for Pro is replaced by codon AAC for Asn,

115th codon CCC for Pro is replaced by codon ACC for Thr,

115th codon CCC for Pro is replaced by codon GGC for Gly,

115th codon CCC for Pro is replaced by codon TAC for Tyr,

115th codon CCC for Pro is replaced by codon GTC for Val,

115th codon CCC for Pro is replaced by codon GAG for Glu,

115th codon CCC for Pro is replaced by codon ATG for Met,

115th codon CCC for Pro is replaced by codon ATC for Ile,

115th codon CCC for Pro is replaced by codon GAC for Asp,

115th codon CCC for Pro is replaced by codon TGG for Trp,

115th codon CCC for Pro is replaced by codon CTC for Leu,

115th codon CCC for Pro is replaced by codon AAG for Lys,

115th codon CCC for Pro is replaced by codon CGC for Arg,

117th codon TAT for Tyr is replaced by codon CAT for His,

118th codon CTG for Leu is replaced by codon CAG for Gln,

131st codon AGC for Ser is replaced by codon ATC for Ile,

132nd codon GCT for Ala is replaced by codon ACT for Thr,

141st codon GAC for Asp is replaced by codon TAC for Tyr,

143rd codon GCC for Ala is replaced by codon GTC for Val,

144th codon GAG for Glu is replaced by codon AAG for Lys,

145th codon TCT for Ser is replaced by codon TGT for Cys,

146th codon GGG for Gly is replaced by codon GAG for Glu, or

153rd codon ATT for Ile is replaced by codon CTG for Leu.

(b) DNAs having the base sequence of formula [A] in which codon GGC encoding 67th Cys and/or 99th Cys is replaced by codon TCT encoding Ser and that codon CCC encoding 115th Pro is replaced by a codon encoding the other amino acid mentioned above.

(c) DNAs resulting from deletion of one or at most 8 successive codons from the 5'-terminus of the above DNAs.

(d) DNAs above in which a translation initiation codon ATG is joined to the 5'-terminus, and/or a termination codon is joined to the 3'-terminus.

The DNAs encoding the novel human TNF polypeptide mutants of the invention can be produced by preparing DNA encoding human TNF or its precursor by a known

method, such as the method described in European Patent Publication No. 155549 or a method of chemical synthesis, and then preparing DNAs encoding the above mutants by point mutation of the resulting DNA in accordance with the method of Wang et al. [Science, 224, 1431 (1984)], or preparing DNAs encoding the above mutants by partial replacement of the resulting DNA using suitable restriction endonucleases and synthetic oligodeoxyribonucleotide adapters in which the base sequence at the desired site(s) is artificially altered.

For example, DNA encoding a polypeptide mutant of formula [I] in which the 115th amino acid (Pro) is replaced by Leu can be produced by the following procedure.

DNA having a base sequence encoding human TNF precursor is isolated by the method described in European Patent Publication No. 155549. The base sequence of DNA encoding the human TNF precursor is shown in Table 8 attached, and a sequence from the 235th base to the 699th base in this base sequence corresponds to a base sequence encoding human TNF. The codon encoding the 115th amino acid (Pro) in the amino acid sequence of human TNF corresponds to the 577th to 579th bases (CCC) in Table 8. A DNA fragment containing this codon is cut out with a combination of suitable restriction endonucleases. Separately, a DNA fragment containing a base sequence resulting from replacing the codon (CCC) for Pro in the above DNA fragment by codon (CTC) for Leu is chemically synthesized. By substituting the synthesized DNA fragment for the cut out DNA fragment, DNA encoding the above polypeptide mutant can be produced.

More specifically, A DNA fragment corresponding to the 555th to 603rd bases in Table 8 is cut out by using restriction endonucleases DdeI and PvuII, for example.

On the other hand, the oligodeoxyribonucleotide adapters having the following base sequences are chemically synthesized.

5'-TGAGGCCAAGCCCTGGTATGAGCTCAT-3'
3'-CCGGTTCGGGACCATACTCGA-5'

and

5'-CTATCTGGGAGGGGTCTTCCAG-3'
3'-GTAGATAGACCCTCCCCAGAAGGTC-5'

The resulting DNA adapters are substituted for the cut out DNA fragment corresponding to the 555th to 603rd bases in Table 8.

By inserting the DNA into a suitable expression vector so that it has a suitable sequence, introducing the vector into a suitable host, and culturing the resulting transformant by techniques known in the art, human TNF polypeptide mutants of the invention can be produced. More specifically, an expression vector for production of the polypeptide mutant of the invention can be produced by preparing a DNA fragment having a translation initiation codon ATG at the 5'-terminus and a termination codon at the 3'-terminus in the DNA having a base sequence encoding the polypeptide mutant itself of the invention, joining the DNA fragment following a suitable promoter and the SD sequence, and then inserting the resulting fragment into a vector. Examples of the promoter are lac, trp, tac, phoS, phoA, PL, and SV40 early promoter. Examples of the vector are plasmids (e.g., pBR322), phages (e.g., lambda phage derivatives), and viruses (e.g., SV40). Transformant can be obtained by introducing the resulting expression vector for production of the polypeptide mutant of the invention into a suitable host, for example, E. coli, by the method of Cohen et al. [Proc. Natl. Acad. Sci., USA, 69, 2110 (1972)]. Then, by culturing the transformant under suitable culturing conditions, the desired polypeptide mutant or one in which Met is joined to its N-terminus can be produced. The cultured cells are treated by, for example, lysozyme digestion, freeze-thawing, ultrasonic rupture, or by using a French press, and then centrifuged or filtered to obtain an extract containing the polypeptide mutant of

the invention. The desired polypeptide mutant can be isolated by purifying the extract in accordance with a general method of purifying proteins (such as ultrafiltration, dialysis, ion exchange chromatography, gel filtration, electrophoresis and affinity chromatography).

The reaction of an organic or inorganic acid or a base with the polypeptide mutant of the invention can give its salt.

Human TNF polypeptide mutants of the invention will be described below in more detail with reference to experimental examples.

(1) Various human TNF polypeptide mutants were produced by culturing the transformants obtained in Examples and Referential Examples given hereinbelow.

Specifically, the transformants were cultured by the method shown in Example 1-(2), and the various human TNF polypeptide mutants produced in the E. coli cells were extracted into 50 mM Tris-HCl buffer (pH 8) containing 0.1% lysozyme and 30 mM NaCl.

The amounts of the desired human TNF polypeptide mutants recovered in the extracts were measured by EIA (enzyme immunoassay) as the amount of the polypeptide which reacted immunologically with an anti-human TNF antibody. The method of determination of the human TNF polypetide mutant according to EIA is based on the following principle.

A competitive binding reaction for an anti-human TNF rabbit antiserum was done between the human TNF polypeptide mutant in an assay sample and human TNF labelled with beta-galactosidase. Then, by adding anti-rabbit IgG goat antiserum insolubilized by binding to a bacterial cell wall, a complex of enzyme-labelled human TNF/anti-human TNF rabbit antibody/anti-rabbit IgG goat antibody was formed. The reaction mixture was centrifuged to obtain a solid phase. The amount of the enzyme-labelled

human TNF in the above complex which was recovered in the solid phase was determined by using its enzyme activity as an index.

Specifically, 2-nitrophenyl-beta-D-galactopyranoside was used as an enzyme substrate, and the amount of the digested product (2-nitrophenol) of the substrate formed by the enzyme reaction was determined by the absorbance at a wavelength of 410 nm. The amount of the enzyme-labelled human TNF in the complex reflects the amount of the human TNF polypeptide mutant in the assay sample.

The amount of the human TNF polypeptide mutant in the assay sample was determined by using a standard curve prepared separately by using human TNF.

In the preparation of the anti-human TNF rabbit antiserum, pure human TNF produced by the method of Yamada et al. [J. Biotechnology, 3, 141 (1985)] was used as an antigen.

The results are shown in Table 3.

When the amount of the desired human TNF polypeptide mutant detected in the cell extract is nearly comparable to that of human TNF used as a control, the solubility of the polypeptide mutant is expressed as (++). Its solubility is expressed as (+) when its detected amount is smaller than the control, and as (-) when it is much smaller than the control or the desired polypeptide mutant is not detected.

It is presumed that the polypeptide mutants having solubilities expressed as (-) underwent structural change and thus markedly decreased in solubility, or were unstable in the E. coli cells.

Table 3

Solubilities of Human TNF Polypeptide Mutants:

Poly-peptide mutant	Mutation: position		solubility
TNF-12T	12th	Ala→Thr	(-)
TNF-13Y	13th	His→Tyr	(-)
TNF-14A	14th	Val→Ala	(-)
TNF-16V	16th	Ala→Val	(++)
TNF-17T	17th	Asn→Thr	(+)
TNF-24F	24th	Leu→Phe	(+)
TNF-26R	26th	Trp→Arg	(-)
TNF-31T	31st	Ala→Thr	(++)
TNF-32A	32nd	Asn→Ala	(++)
TNF-32C	32nd	Asn→Cys	(++)
TNF-32D	32nd	Asn→Asp	(++)
TNF-32H	32nd	Asn→His	(++)
TNF-32I	32nd	Asn→Ile	(++)
TNF-32R	32nd	Asn→Arg	(++)
TNF-32S	32nd	Asn→Ser	(++)
TNF-32T	32nd	Asn→Thr	(++)
TNF-32V	32nd	Asn→Val	(++)
TNF-32Y	32nd	Asn→Tyr	(++)
TNF-32G	32nd	Asn→Gly	(++)
TNF-32L	32nd	Asn→Leu	(++)
TNF-34I	34th	Leu→Ile	(++)
TNF-35P	35th	Leu→Pro	(-)
TNF-36V	36th	Ala→Val	(++)
TNF-44D	44th	Asn→Asp	(-)
TNF-45P	45th	Gln→Pro	(-)
TNF-48M	48th	Val→Met	(++)
TNF-50P	50th	Ser→Pro	(-)
TNF-54C	54th	Tyr→Cys	(-)
TNF-54H	54th	Tyr→His	(-)

- to be continued -

Table 3 (continued)

Poly-peptide mutant	Mutation: position		solubility
TNF-58P	58th	Ser→Pro	(-)
TNF-59L	59th	Gln→Leu	(-)
TNF-60D	60th	Val→Asp	(-)
TNF-60G	60th	Val→Gly	(-)
TNF-62S	62nd	Phe→Ser	(-)
TNF-67S	67th	Cys→Ser	(++)
TNF-70Y	70th	Thr→Tyr	(++)
TNF-73P	73rd	Leu→Pro	(++)
TNF-82D	82nd	Ala→Asp	(++)
TNF-85H	85th	Tyr→His	(++)
TNF-89I	89th	Val→Ile	(++)
TNF-93P	93rd	Ser→Pro	(-)
TNF-94T	94th	Ala→Thr	(++)
TNF-97N	97th	Ser→Asn	(++)
TNF-98H	98th	Pro→His	(++)
TNF-98L	98th	Pro→Leu	(++)
TNF-99S	99th	Cys→Ser	(++)
TNF-103P	103rd	Thr→Pro	(++)
TNF-113C	113th	Tyr→Cys	(++)
TNF-115H	115th	Pro→His	(++)
TNF-115Q	115th	Pro→Gln	(++)
TNF-115S	115th	Pro→Ser	(++)
TNF-115A	115th	Pro→Ala	(++)
TNF-115F	115th	Pro→Phe	(+)
TNF-115N	115th	Pro→Asn	(++)
TNF-115T	115th	Pro→Thr	(++)
TNF-115G	115th	Pro→Gly	(++)
TNF-115Y	115th	Pro→Tyr	(++)
TNF-115V	115th	Pro→Val	(++)
TNF-115E	115th	Pro→Glu	(++)

- to be continued -

Table 3 (continued)

Poly-peptide mutant	Mutation: position		solubility
TNF-115M	115th	Pro→Met	(+)
TNF-115I	115th	Pro→Ile	(++)
TNF-115D	115th	Pro→Asp	(++)
TNF-115W	115th	Pro→Trp	(++)
TNF-115L	115th	Pro→Leu	(++)
TNF-115K	115th	Pro→Lys	(++)
TNF-115R	115th	Pro→Arg	(+)
TNF-117H	117th	Tyr→His	(++)
TNF-118Q	118th	Leu→Gln	(++)
TNF-121G	121st	Val→Gly	(-)
TNF-124Q	124th	Leu→Gln	(-)
TNF-128A	128th	Asp→Ala	(-)
TNF-128N	128th	Asp→Asn	(-)
TNF-131I	131st	Ser→Ile	(++)
TNF-132T	132nd	Ala→Thr	(++)
TNF-135D	135th	Asn→Asp	(+)
TNF-138Y	138th	Asp→Tyr	(+)
TNF-141Y	141st	Asp→Tyr	(++)
TNF-143V	143rd	Ala→Val	(++)
TNF-144K	144th	Glu→Lys	(++)
TNF-145C	145th	Ser→Cys	(++)
TNF-146E	146th	Gly→Glu	(++)
TNF-148D	148th	Val→Asp	(-)
TNF-148G	148th	Val→Gly	(-)
TNF-150L	150th	Phe→Leu	(-)
TNF-151E	151st	Gly→Glu	(-)
TNF-153L	153rd	Ile→Leu	(++)
TNF-115L-Ser67			(++)
TNF-115LΔN8-Ser67			(++)
TNF-115LΔN8			(++)

(2) Table 4 shows the isoelectric points and cytotoxic activities of human TNF (as a control) and the various human TNF polypeptide mutants of the invention obtained in Examples given hereinbelow.

The cytotoxic activity was evaluated on mouse L-M cells (ATCC, CCL 1.2) by the method of Yamada et al. [J. Biotechnology, 3, 141 (1985)].

The human TNF polypeptide mutants which were determined to be homogeneous from SDS-polyacrylamide gel electrophoretic analysis [U. K. Laemmli, Nature (London), 227, 680 (1970)] were used in this test.

Table 4

Properties of Human TNF Polypeptide Mutant: Isoelectric point and cytotoxic activity

Mutant polypeptide	Isoelectric point (pI)	Cytotoxic activity (U/μg)
Human TNF	5.9	2,080
TNF-16V	5.7	310
TNF-31T	5.8	12
TNF-32Y	5.9	0.18
TNF-32H	6.1	32
TNF-32D	5.5	1.1
TNF-32S	5.8	1.0
TNF-32G	5.8	8.7
TNF-32L	5.8	0.43
TNF-36V	5.9	122
TNF-73P	6.0	234
TNF-98H	6.4	1,330
TNF-103P	6.2	215
TNF-115L	5.9	12
TNF-115S	5.8	23
TNF-115T	5.8	38
TNF-115H	6.0	220
TNF-115R	7.0	0.22
TNF-115D	5.7	6.8
TNF-115G	5.9	37
TNF-117H	6.3	31
TNF-131I	5.9	1,890
TNF-143V	5.8	243
TNF-144K	7.5	1.3
TNF-146E	5.6	0.18
TNF-115L$^{\Delta}$N8	5.9	10

(3) Table 5 shows the antitumor activities *in vivo* of human TNF (as a control) and the various human TNF polypeptide mutants obtained in Examples given hereinbelow. The antitumor activity was evaluated as follows:-

Meth A sarcoma cells (2×10^5) were transplanted into the abdominal skin of BALB/c female mice (8 week old). Seven days after the transplantation, the polypeptide was administered once intravenously. The tumor necrotizing response was evaluated 24 hours after the administration by the evaluation standards of Carswell et al. [Proc. Natl. Acad. Sci. USA, 72, 3666 (1975)].

As shown in Table 5, the correlation between the cytotoxic activity *in vitro* and the antitumor activity *in vivo* against the transplanted tumor is scarce. For example, TNF-131I has nearly the same *in vitro* cytotoxic activity and *in vivo* antitumor activity as those of human TNF. However, other human TNF polypeptide mutants, for example human TNF polypeptide mutant in which the 32nd, 115th or 143rd amino acid from the N-terminus is replaced, show strong *in vivo* antitumor activity as compared with their *in vitro* cytotoxic activity, and have low lethal toxicity.

Table 5

Antitumor Effect of Human TNF Polypeptide Mutant on Meth A Sarcoma Transplanted in Mice:

Polypeptide: dose (unit)/mouse	Necrotic Response -	+	++	+++	Inhibition of Tumor Growth (%)
Human TNF:					
600	1	5	0	0	55
2,000	0	4	2	0	80
6,000	0	0	6	0	87
20,000	0	0	0	6	100
TNF-16V:					
93	6	0	0	0	61
310	4	2	0	0	64
930	0	4	2	0	90
3,100	0	0	6	0	86
9,300	0	0	3	3	100
TNF-31T:					
35	7	0	0	0	49
115	2	5	0	0	79
TNF-32Y:					
5	4	3	0	0	26
18	3	4	0	0	46
TNF-32D:					
11	4	0	0	0	63
33	0	5	0	0	82
110	0	1	3	1	81
TNF-32S:					
10	5	0	0	0	17
30	1	4	0	0	68
100	0	1	4	0	97
TNF-32H:					
32	4	1	0	0	45
96	1	4	0	0	73
320	0	3	2	0	100

- to be continued -

Table 5 (continued)

Polypeptide: dose (unit)/mouse	Necrotic Response -	+	++	+++	Inhibition of Tumor Growth (%)
TNF-36V:					
120	2	4	0	0	49
370	0	4	2	0	83
1,200	0	0	2	4	93
3,700	0	0	0	6	93
TNF-73P:					
230	5	2	0	0	8
700	0	7	0	0	66
2,300	0	5	2	0	90
TNF-98H:					
1,300	2	5	0	0	70
4,000	0	5	2	0	81
13,000	0	0	1	6	94
TNF-103P:					
215	0	1	6	0	70
645	0	0	4	3	100
TNF-115L:					
12	5	1	0	0	70
35	2	4	0	0	73
117	0	5	1	0	78
350	0	2	4	0	91
1,170	0	0	5	1	93
TNF-115S:					
23	3	3	0	0	74
69	0	4	2	0	81
230	0	2	4	0	91
690	0	0	5	1	84
2,300	0	0	1	5	100
TNF-115H:					
220	5	1	0	0	72
660	0	5	1	0	80
2,200	0	1	5	0	96

- to be continued -

Table 5 (continued)

Polypeptide: dose (unit)/mouse	Necrotic Response -	+	++	+++	Inhibition of Tumor Growth (%)
TNF-115T:					
38	2	3	0	0	54
114	2	3	0	0	83
380	1	2	2	0	91
1,140	0	0	5	0	100
TNF-115D:					
68	0	5	0	0	57
204	0	2	3	0	100
TNF-115G:					
110	0	5	0	0	68
370	0	1	4	0	98
1,100	0	0	1	4	100
TNF-117H:					
31	4	3	0	0	51
92	1	6	0	0	88
310	0	0	7	0	93
TNF-131I:					
5,700	0	0	2	3	83
19,000	0	0	1	3	98
TNF-143V:					
730	0	5	0	0	42
2,400	0	4	1	0	65
7,300	0	1	3	1	100

(4) Several human TNF polypeptide mutants of this invention as well as human TNF were tested for pyrogenicity in rabbit. The results are shown in Table 6.

The pyrogenicity test was carried out by administering the polypeptide intravenously to rabbit, and observing change in the rectal temperature for 4 hours after the administration. The results are expressed as follows:

(-): a rectal temperarure rise of not more than $0.4^{\circ}C$

(+): a rectal temperature rise of 0.5 to $0.9^{\circ}C$

(++): a rectal temperature rise of $1.0^{\circ}C$ or more

Table 6

Pyrogenicity of Human TNF Polypeptide Mutant in Rabbit:

Polypeptide:	dose (μg/kg)	Pyrogenicity
Human TNF:	0.50	(+)
TNF-16V:	0.52	(-)
	5.2	(-)
	52	(+)
TNF-32Y:	0.56	(-)
	5.6	(-)
	56	(-)
TNF-32H:	0.50	(-)
	5.0	(-)
	50	(-)
TNF-36V:	0.50	(-)
	5.0	(+)
	50	(+)
TNF-73P:	0.51	(-)
	5.1	(+)
	51	(++)
TNF-115L:	0.67	(-)
	6.7	(-)
	67	(+)
TNF-115S:	0.52	(-)
	5.2	(+)
	52	(+)
TNF-115H:	0.50	(-)
	5.0	(+)
	50	(+)
TNF-117H:	0.52	(-)
	5.2	(-)
	52	(+)

(5) The effect of the polypeptide mutant (TMF-115L) on the blood pressure was tested by administering into the tail vein of SHR/NCrj male rats (body weight 264 to 304 g; Nippon Charles River Co., Ltd.), and measuring the systolic blood pressure of the rats without anesthesia by means of an arterial pressure measuring device for rats (Model KN-209, made by Natsume Seisakusho). As a control human TNF was administered as well.

The results are shown in Table 7.

Table 7

Effect of TNF-115L on Blood Pressure in Rats

Polypeptide dosage (μg/kg)	Changes in Blood Pressure (mean±SD) (hours after Administration) before	5 hours	24 hours
Human TNF: 100	193±3.1 mmHg	183±2.0 mmHg	170±3.2 mmHg
TNF-115L: 100	189±3.0 mmHg	195±2.5 mmHg	191±2.6 mmHg
1,000	189±3.0	178±2.7	184±2.2
5,000	190±1.5	186±2.1	189±2.5
10,000	191±2.1	185±2.4	188±3.6

For formulating human TNF polypeptide mutants of this invention, they may be in the form of a solution or a lyophilized product. From the standpoint of long-term stability, they are desirably in the form of lyophilized products. It is preferred to add vehicles or stabilizers to the preparations. Examples of the stabilizers include albumin, globulin, gelatin, protamine, protamine salts, glucose, galactose, xylose, mannitol, glucuronic acid, trehalose, dextran, hydroxyethyl starch, and nonionic surface-active agents (such as polyoxyethylene fatty acid esters, polyoxyethylene alkyl ethers, polyoxyethylene alkyl phenyl ethers, polyoxyethylene sorbitan fatty acid esters, polyoxyethylene glycerin fatty acid esters, polyoxyethylene hardened castor oil, polyoxyethyene castor oil, polyoxyethylene polyoxypropylene alkyl ethers, polyoxyethylene polyoxypropylene block copolymer, sorbitan fatty acid esters, sucrose fatty acid esters and glycerin fatty acid esters).

The several human TNF polypeptide mutants of this invention are especially useful as antitumor agents as shown above.

Such polypeptide preparations are preferably administered parenterally or topically. Parenteral routes such as intraveous and intramuscular routes are used when tumor cells extend over a wide range or metastasize, or when prevention of metastasis is intended. Against local tumor tissues, direct intratumor administration is preferred. The dosage varies depending upon the type of human TNF polypeptide mutants and the type and size of tumors, the condition of the patient and the route of administration. For example, in case of TNF-115L, it is 1 x 10^3 to 1 x 10^8 units (LM)/kg, preferably 1 x 10^4 to 1 x 10^7 units (LM)/kg.

The following examples illustrate the present invention more specifically. It should be understood

however that other human TNF polypeptide mutants in accordance with this invention can also be produced by similar methods, and the invention is in no way limited to these examples.

Example 1

Production of Human TNF Polypeptide Mutant TNF-32Y:-

(1) Construction of an expression plasmid

An expression plasmid (pHNY-32) for producing a polypeptide consisting of 155 amino acids corresponding to the sequence from amino acid No. 1 to No. 155 in Table 9 attached, referred to as TNF-32Y, was constructed as illustrated in Figures 2 and 3.

A cloned cDNA encoding human TNF was isolated by digestion with restriction endonuclease PstI from the recombinant plasmid pHTNF13 prepared according to the method described in European Patent Publication No. 155549.

The cloned cDNA was further digested with restriction endonucleases AvaI and HindIII to isolate a DNA fragment containing most of the coding region for the human TNF polypeptide. The isolated DNA fragment is referred to as TNF-DNA fragment.

The TNF-DNA fragment was about 600 bp in size containing the base sequence corresponding to the downstream region from base No. 250 in Table 8. Its full base sequence was reported by Yamada et al. [J. Biotechnology, 3, 141 (1985)].

The TNF-DNA fragment was further digested with restriction endonucleases HpaII and BglI to cut it into three DNA fragments and they were isolated. These DNA fragments had the sequences corresponding to the region from base No. 250 to No. 321, the region from base No. 322 to 337 and the downstream region from base No. 338 in Table 8, respectively. These DNA fragments were named DNA-1 fragment, DNA-2 fragment and DNA-3 fragment, respectively.

Then, the DNA-1 and DNA-3 fragments were combined by using T4 DNA ligase with the following chemically synthesized oligodeoxyribonucleotide adapter [a].

```
5'-CGGGCCTATGCCCTCC-3'          [a]
  3'-CCGGATACGGG-5'
```

The ligated DNA fragment is referred to as NY-DNA fragment. The NY-DNA fragment was sequentially ligated with the following two chemically synthesized oligodeoxyribonucleotide adapters, [b] and [c].

```
5'-AACTAGTACGCAAGTTCACGTAAGGAGGTTATC-3'      [b]
3'-TTGATCATGCGTTCAAGTGCATTCCTCCAATAGCTA-5'
```

and

```
5'-GATTATGTCATCTTCTCGAACC-3'          [c]
   3'-ATACAGTAGAAGAGCTTGGGGCT-5'
```

The resulting DNA fragment is referred to as Peptide coding-DNA fragment.

A DNA fragment (about 380 bp in size) containing the trp promoter region was isolated from a plasmid pCT-1 [M. Ikehara et al., Proc. Natl. Acad. Sci., USA, 81, 5956 (1984)] by double digestion with restriction endonucleases HpaI and AatII. The base sequence of the trp promoter region of the above 380 bp-DNA fragment was reported by Bennett et al. [J. Mol. Biol., 121, 113 (1978)]. The above 380 bp-DNA fragment was ligated with the Peptide coding-DNA fragment prepared as above. The ligated DNA fragment was referred to as Promoter-Peptide coding-DNA fragment.

Separately, a plasmid pBR322 was digested with restriction endonucleases AvaI and PvuII, and the resulting larger DNA fragment (about 3.7 kbp in size) was isolated by 0.7% agarose gel electrophoresis. After filling-in its cohesive ends to blunt ends with E. coli

DNA polymerase I (Klenow fragment) and four kinds of deoxyribonucleotide triphosphates (dGTP, dATP, dTTP and dCTP), both ends were ligated by T4 DNA ligase to construct a new plasmid, which is designated pBRS6.

The plasmid pBRS6 was cleaved with restriction endonucleases AatII and HindIII into two DNA fragments. The larger DNA fragment (about 3.6 kbp in size) was isolated and ligated by T4 DNA ligase with the Promoter-Peptide coding-DNA fragment prepared as above in order to construct an expression plasmid pHNY-32.

(2) Production of TNF-32Y

The expression plasmid pHNY-32 was introduced into E. coli HB101 by the conventional method [S. N. Cohen et al., Proc. Natl. Acad. Sci., USA, 69, 2110 (1972)].

The transformant (HB101/pHNY-32) was cultivated at 37°C overnight in LB broth [composition: 1% trypton, 0.5% yeast extract, 1% NaCl, pH 7.5]. The culture was inoculated in 10-volumes of modified M9 medium [composition: 1.5% $Na_2HPO_4 \cdot 12H_2O$, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl, 2 mg/liter of vitamine B1, 0.45% casamino acid, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 0.4% glycerol] containing ampicillin at 25 micrograms/ml, at 37°C for 1 hour.

Then, 3-indoleacrylic acid was added to give a final concentration of 20 micrograms/ml. After the cultivation was further continued for 24 hours, the cells were collected by centrifugation. The cells were suspended in 50 mM Tris-HCl buffer (pH 8) containing 0.1% lysozyme and 30 mM NaCl, and allowed to stand in an ice bath for 30 minutes. After the cell suspension was repeatedly treated by freezing in a dry ice/ethanol bath and thawing at 37°C, the cell extract was collected by centrifugation.

The cell extract was dialyzed against 20 mM Tris-HCl buffer (pH 7.8), and the dialyzate was centrifuged to obtain a clarified supernatant. The supernatant was applied onto a DEAE-Sepharose CL-6B column (Pharmacia)

previously equilibrated with 20 mM Tris-HCl buffer (pH 7.8). After the column was washed with the same buffer to remove non-adsorbed components, the desired polypeptide (TNF-32Y) was eluted with a linear gradient of NaCl concentration from zero to 0.3 M in the same buffer. Each fraction was subjected to SDS-polyacrylamide gel electrophoresis, and the fractions containing polypeptide having a molecular weight of about 17 kilodaltons were collected and pooled.

The pooled fraction was dialyzed against 20 mM Tris-HCl buffer (pH 7.8), and then it was again subjected to DEAE-Sepharose CL-6B column chromatography as above, but the elution was carried out under the elution condition of an easier gradient of NaCl concentration.

The fractions containing the desired polypeptide were collected, pooled and concentrated by ultrafiltration with Diaflo using a YM10 membrane (Amicon).

Finally, the concentrate was subjected to gel filtration on a column of Bio-Gel P-6 (Bio-Rad) using 5 mM phosphate buffered saline as an eluent to obtain purified TNF-32Y.

N-terminal amino acid sequence of the purified TNF-32Y was analyzed by the automated Edman degradation on Protein Sequencer (Applied Biosystems, Model 470A).

As a result, the N-terminal amino acid of TNF-32Y was a serine residue. Namely, a methionine residue due to the translation initiation codon (ATG) was removed from the purified product.

Example 2

Production of Human TNF Polypeptide Mutant TNF-115L:-

(1) Construction of an expression plasmid

An expression plasmid (pHPL-115) for producing a polypeptide consisting of 155 amino acids corresponding to the sequence of the amino acid No. 1 to No. 155 in Table 10 attached, referred to as TNF-115L, was constructed as illustrated in Figure 4.

The TNF-DNA fragment prepared as mentioned in Example 1-(1) was digested with restriction endonucleases PvuII and TaqI to cut it into four DNA fragments and they were isolated. These DNA fragments had the sequences corresponding to the region from base No. 250 to 369, the region from base No. 370 to 603, the region from base No. 604 to No. 653 and the downstream region from base No. 654 in Table 8, respectively.

These DNA fragments were named DNA-4 fragment, DNA-5 fragment, DNA-6 fragment and DNA-7 fragment, respectively. The DNA-5 fragment was further digested with restriction endonuclease DdeI to isolate a DNA fragment corresponding to the sequence from base No. 370 to No. 554 in Table 8 (referred to as DNA-8 fragment).

The DNA-8 fragment was combined with the DNA-4 fragment, and then ligated with the following two chemically synthesized oligodeoxyribonucleotide adapters, [d] and [e].

```
5'-TGAGGCCAAGCCCTGGTATGAGCTCAT-3'        [d]
   3'-CCGGTTCGGGACCATACTCGA-5'
```

and

```
   5'-CTATCTGGGAGGGGTCTTCCAG-3'          [e]
3'-GTAGATAGACCCTCCCCAGAAGGTC-5'
```

To the ligated DNA fragment, the DNA-6 fragment and the DNA-7 fragment were further ligated by using T4 DNA ligase. The resulting DNA fragment is referred to as PL-DNA fragment.

The expression plasmid pHPL-115 was constructed according to the method as mentioned in Example 1-(1), except for using the PL-DNA fragment instead of the NY-DNA fragment.

(2) Production of TNF-115L

According to the method mentioned in Example 1-(2), the transformant (HB101/pHPL-115) was prepared and cultivated. The desired polypeptide was isolated and purified from the cell extract according to essentially the same method as mentioned in Example 1-(2).

(3) Determination of amino acid sequence

Amino acid sequences of the purified TNF-115L and its peptide fragment were analyzed by the automated Edman degradation on a Protein Sequencer.

The peptide fragment was prepared under the following conditions. Five hundred micrograms of the purified TNF-115L was incubated with 10 micrograms of lysyl endopeptidase (EC 3.4.21.50: Wako Pure Chemical Ind.) in 5 mM Tric-HCl buffer (pH 8) containing 4M urea in a total volume of 0.1 ml. After incubation at 35°C for 15 hours, the resulting digested peptides were isolated by high performance liquid chromatography using a column of SynChropak RP-P300 (250 x 4.6 mm; SynChrom Inc.) under the conditions of a linear gradient elution from 10% to 50% of acetonitrile containing 0.07% trifluoroacetic acid, in 0.1% trifluoroacetic acid, at a flow rate of 1 ml/min for 60 minutes. The elution pattern is shown in Figure. 5. The peptide fragments were isolated from each of fractions of No. 1 to No. 7 in Figure 5 and subjected to analysis of amino acid sequence by the automated Edman degradation method.

The partial amino acid sequence of peptide fragment No. 6 was determined to be Pro-X-Tyr-Glu-Leu-Ile-Tyr-Leu-Gly-Gly-Val-Phe-Gln-Leu-Glu. Mark "X" shows an amino acid which could not be determined by this analysis.

The determined amino acid sequence as above was completely agreed with the sequence from amino acid No. 111 to No. 125 in Table 10.

It was confirmed that the amino acid at the 115th position from the N-terminus of TNF-115L was a leucine residue.

N-terminal amino acid of the purified TNF-115L was a serine residue, indicating that a methionine residue due to the translation initiation codon (ATG) was removed.

Example 3

Production of Human TNF Polypeptide Mutant TNF-115LΔN8:-

(1) Construction of an expression plasmid

An expression plasmid (pHPL-147) for producing a polypeptide consisting of 147 amino acids corresponding to the sequence from amino acid No. 9 (Lys) to No. 155 (Leu) in Table 10, referred to as TNF-115LΔN8, was constructed as illustrated in Figure 6.

The expression plasmid pHPL-115 prepared as mentioned in Example 2-(1) was cut into two DNA fragments by digestion with restriction endonucleases ClaI and BstEII. The larger fragment contains the downstream region from base No. 380 in Table 10 (coding region for C-terminal portion of TNF-115L), tetracycline-resistance gene, ampicillin-resistance gene and the trp promoter region, which is referred to as Vector-DNA fragment.

The smaller fragment contains the region corresponding to the sequence from base No. 1 to No. 379 in Table 10. This fragment was further digested with restriction endonuclease HgiAI to isolate a DNA fragment corresponding to the sequence from the base No. 219 to No. 379, which is referred to as Hgi-DNA fragment.

Separately, the expression plasmid pHT147 prepared as mentioned in Referential Example 2, was digested with restriction endonucleases ClaI and HgiAI to isolate a DNA fragment (about 200 bp in size) containing the region corresponding to the sequence from base No. 25 to No. 218 in Table 10. This DNA fragment is referred to as ΔN8-DNA fragment.

The ΔN8-DNA fragment was ligated with the Hgi-DNA fragment by using T4 DNA ligase, and then the ligated DNA fragment was combined with the Vector-DNA fragment prepared as above to construct the expression plasmid pHPL-147.

(2) Production of TNF-115LΔN8

According to the method as mentioned in Example

1-(2), the transformant (HB101/pHPL-147) was prepared and cultivated. The desired polypeptide was isolated and purified from the cell extract according to essentially the same method as mentioned in Example 1-(2).

At the N-terminus of purified TNF-115LΔN8, a methionine residue due to the translation initiation codon (ATG) was detected by the automated Edman degradation method.

Example 4

Production of Human TNF Polypeptide Mutant TNF-115L-Ser67:-

(1) Construction of an expression plasmid

An expression plasmid (pHPL-Ser67) for producing a polypeptide consisting of 155 amino acids and having an amino acid sequence corresponding to the sequence from amino acid No. 1 to No. 155 in Table 10, in which a cysteine residue at amino acid No. 67 was replaced by a serine residue, referred to as TNF-115L-Ser67, was constructed as illustrated in Figure 7.

The expression plasmid pHTP392 prepared as mentioned in Referential Example 3 was digested with restriction endonucleases ClaI, HgiAI and HpaI to isolate a DNA fragment (about 226 bp in size) containing the base sequence from base No. 1 to No. 218 in Table 10, but in which two bases at base Nos. 200 and 201 in Table 10, G and C, were replaced by C and T, respectively. The DNA fragment is referred to as Ser67-DNA fragment.

The Ser67-DNA fragment was ligated with the Hgi-DNA fragment prepared as mentioned in Example 3-(1), and the ligated DNA fragment was combined with the Vector-DNA fragment prepared as mentioned in Example 3-(1), in order to construct the expression plasmid pHPL-Ser67.

(2) Production of TNF-115L-Ser67

According to the method as mentioned in Example 1-(2), the transformant (HB101/pHPL-Ser67) was prepared and cultivated. The desired polypeptide was isolated and

purified from the cell extract by essentially the same method as mentioned in Example 1-(2).

A methionine residue due to the translation initiation codon (ATG) was not detected at the N-terminus of purified TNF-115L-Ser67 by the automated Edman degradation method. The N-terminal amino acid was serine residue.

Example 5

Production of Human TNF Polypeptide Mutant TNF-115LΔN8-Ser67:-

(1) Construction of an expression plasmid

An expression plasmid (pHPL147S67) for producing a polypeptide consisting of 147 amino acids and having an amino acid sequence corresponding to the sequence from amino acid No. 9 to No. 155 in Table 10, in which a cysteine residue in amino acid No. 67 was replaced by a serine residue, referred to as TNF-115LΔN8-Ser67, was constructed as illustrated in Figure 8.

The expression plasmid pHPL-Ser67 prepared as mentioned in Referential Example 4-(1) was digested with restriction endonucleases ClaI and BstEII to cleave two fragments. The larger DNA fragment is the same fragment with the Vector-DNA fragment prepared in Example 3. The smaller DNA fragment was further digested with restriction endonuclease RsaI to isolate a DNA fragment containing the base sequence from base No. 161 to No. 379 in Table 10, which is referred to as Rsa-DNA fragment.

Separately, the expression plasmid pHPL-147 prepared as mentioned in Example 3-(1) was digested with restriction endonucleases ClaI and RsaI to isolate a DNA fragment (about 144 bp in size) containing the base sequence from base No. 25 to No. 160 in Table 10.

The 144 bp-DNA fragment prepared as above was ligated with the Rsa-DNA fragment by T4 DNA ligase, and the resulting DNA fragment was combined with the Vector-

DNA fragment to construct the expression plasmid, pHPL147S67, for producing TNF-115LΔN8-Ser67.

(2) Production of TNF-115LΔN8-Ser67

According to the method as mentioned in Example 1-(2), the transformant (HB101/pHPL147S67) was prepared and cultivated. The desired polypeptide was isolated from the cell extract and purified according to essentially the same method as mentioned in Example 1-(2).

A methionine residue due to the translation initiation codon (ATG) was detected at the N-terminus of purified TNF-115LΔN8-Ser67 by the automated Edman degradation method.

Example 6

Production of Other Human TNF Polypeptide Mutants-1:-

(1) Construction of expression plasmids

An expression plasmid for producing a polypeptide consisting of 155 amino acids and having an amino acid sequence corresponding to the sequence from amino acid No. 1 to No. 155 in Table 1, in which an asparagine residue in the 32nd position from the N-terminus was replaced by another amino acid, for example, His, Asp and Ser, was constructed according to the method as mentioned in Example 1-(1), except for using one of the chemically synthesized oligodeoxyribonucleotide adaptors shown below instead of the synthetic adapter [a]:

```
5'-CGGGCCCACGCCCTCC-3'
  3'-CCGGGTGCGGG-5'
```

(for replacement by His),

```
5'-CGGGCCGATGCCCTCC-3'
  3'-CCGGCTACGGG-5'
```

(for replacement by Asp),

or

```
5'-CGGGCCAGCGCCCTCC-3'
  3'-CCGGTCGCGGG-5'
```

(for replacement by Ser).

(2) Production of human TNF polypeptide mutant

Each of the expression plasmids obtained in Section (1) was introduced in E. coli HB101 by the conventional method, and the transformant was cultivated according to the method described in Example 1-(2).

The desired polypeptide was purified from the cell extract by essentially the same method as mentioned in Example 1-(2).

There were obtained the followng human TNF polypeptide mutants.

TNF-32H: Polypeptide having an amino acid sequence of formula [I] in which 32nd Asn was replaced by His.

TNF-32D: Polypeptide having an amino acid sequence of formula [I] in which 32nd Asn was replaced by Asp.

TNF-32S: Polypeptide having an amino acid sequence of formula [I] in which 32nd Asn was replaced by Ser.

Example 7

Production of Other Human TNF Polypeptide Mutants-2:-

(1) Construction of expression plasmids

An expression plasmid for producing a polypeptide consisting of 155 amino acids and having an amino acid sequence corresponding to the sequence from amino acid No. 1 to No. 155 in Table 1, in which a proline residue in the 115th position from the N-terminus was replaced by another amino acid, for example, Ser, Asp and Gly, was constructed according to the method as mentioned in Example 2-(1), except for using one of the chemically synthesized oligodeoxyribonucleotide adapters shown below instead of the synthetic adapter [d]:

5'-TGAGGCCAAGCCCTGGTATGAGTCCAT-3'
3'-CCGGTTCGGGACCATACTCAG-5'

(for replacement by Ser),

5'-TGAGGCCAAGCCCTGGTATGAGGACAT-3'
3'-CCGGTTCGGGACCATACTCCT-5'

(for replacement by Asp),

or

5'-TGAGGCCAAGCCCTGGTATGAGGGCAT-3'
3'-CCGGTTCGGGACCATACTCCC-5'

(for replacement by Gly).

(2) Production of human TNF polypeptide mutants

Each of the expression plasmids obtained in Section (1) was introduced in E. coli HB101 by the conventional method, and the transformant was cultivated according to the method described in Example 1-(2).

The desired polypeptide was isolated and purified from the cell extract by essentially the same method as described in Example 1-(2).

There were obtained the followng human TNF polypeptide mutants.

TNF-115S: Polypeptide having an amino acid sequence of formula [I] in which 115th Pro was replaced by Ser.

TNF-115D: Polypeptide having an amino acid sequence of formula [I] in which 115th Pro was replaced by Asp.

TNF-115G: Polypeptide having an amino acid sequence of formula [I] in which 115th Pro was replaced by Gly.

Example 8

Production of Human TNF Polypeptide Mutant TNF-117H, referred to as TNF-117H:-

(1) Construction of expression plasmids

An expression plasmid for producing a polypeptide consisting of 155 amino acids and having an amino acid sequence corresponding to the sequence from amino acid No. 1 to No. 155 in Table 1, in which a tyrosine

residue in the 117th position from the N-terminus replaced by another amino acid, for example, His, was constructed according to the method as mentioned in Example 2-(1), except for using a chemically synthesized oligodeoxyribonucleotide adapter shown below instead of the synthetic adapter [e]:

5'-CCATCTGGGAGGGGTCTTCCAG-3'
3'-GTAGGTAGACCCTCCCCAGAAGGTC-5'

(2) Production of TNF-117H

The expression plasmid obtained in Section (1) was introduced in E. coli HB101 by the conventional method, and the transformant was cultivated according to the method described in Example 1-(2).

The desired polypeptide was isolated and purified from the cell extract by essentially the same method as mentioned in Example 1-(2).

Example 9

Production of Other Human TNF Polypeptide Mutants-3:-

In accordance with Example 1, expression plasmids for production of the following polypeptides were constructed. Escherichia coli was transformed with the expression plasmids. The transformants were cultured, and the polypeptides were isolated and purified.

TNF-16V: Polypeptide having an amino acid sequence of formula [I] in which 16th Ala was replaced by Val

TNF-31T: Polypeptide having an amino acid sequence of formula [I] in which 31st Ala was replaced by Thr

TNF-32G: Polypeptide having an amino acid sequence of formula [I] in which 32nd Asn was replaced by Gly

TNF-32L: Polypeptide having an amino acid sequence of formula [I] in which 32nd Asn was replaced by Leu

TNF-36V: Polypeptide having an amino acid sequence of formula [I] in which 36th Ala was replaced by Val

TNF-73P: Polypeptide having an amino acid sequence of formula [I] in which 73rd Leu was replaced by Pro

TNF-82D: Polypeptide having an amino acid sequence of formula [I] in which 82nd Ala was replaced by Asp
pI 5.3 (its isoelectoric point was 5.3)

TNF-85H: Polypeptide having an amino acid sequence of formula [I] in which 85th Tyr was replaced by His
pI 6.4 (its isoelectoric point was 6.4)

TNF-98H: Polypeptide having an amino acid sequence of formula [I] in which 98th Pro was replaced by His

TNF-103P: Polypeptide having an amino acid sequence of formula [I] in which 103rd Thr was replaced by Pro

TNF-115T: Polypeptide having an amino acid sequence of formula [I] in which 115th Pro was replaced by Thr

TNF-115H: Polypeptide having an amino acid sequence of formula [I] in which 115th Pro was replaced by His

TNF-115R: Polypeptide having an amino acid sequence of formula [I] in which 115th Pro was replaced by Arg

TNF-131I: Polypeptide having an amino acid sequence of formula [I] in which 131st Ser was replaced by Ile

TNF-141Y: Polypeptide having an amino acid sequence of formula [I] in which 141st Asp was replaced by Tyr

TNF-143V: Polypeptide having an amino acid sequence of formula [I] in which 143rd Ala was replaced by Val

TNF-144K: Polypeptide having an amino acid sequence of formula [I] in which 144th Glu was replaced by Lys

TNF-146E: Polypeptide having an amino acid sequence of formula [I] in which 146th Gly was replaced by Glu

Example 10

Production of Other Human TNF Polypeptide Mutants-4:-

In accordance with Example 1, expression plasmids for production of the following polypeptides were constructed. Escherichia coli was transformed with the expression plasmids. The transformants were cultured to produce the polypeptides.

TNF-32A: Polypeptide having an amino acid sequence of formula [I] in which 32nd Asn was replaced by Ala

TNF-32C: Polypeptide having an amino acid sequence of formula [I] in which 32nd Asn was replaced by Cys

TNF-32I: Polypeptide having an amino acid sequence of formula [I] in which 32nd Asn was replaced by Ile

TNF-32R: Polypeptide having an amino acid sequence of formula [I] in which 32nd Asn was replaced by Arg

TNF-32T: Polypeptide having an amino acid sequence of formula [I] in which 32nd Asn was replaced by Thr

TNF-32V: Polypeptide having an amino acid sequence of formula [I] in which 32nd Asn was replaced by Val

TNF-34I: Polypeptide having an amino acid sequence of formula [I] in which 34th Leu was replaced by Ile

TNF-48M: Polypeptide having an amino acid sequence of formula [I] in which 48th Val was replaced by Met

TNF-89I: Polypeptide having an amino acid sequence of formula [I] in which 89th Val was replaced by Ile

TNF-94T: Polypeptide having an amino acid sequence of formula [I] in which 94th Ala was replaced by Thr

TNF-97N: Polypeptide having an amino acid sequence of formula [I] in which 97th Ser was replaced by Asn

TNF-98L: Polypeptide having an amino acid sequence of formula [I] in which 98th Pro was replaced by Leu

TNF-113C: Polypeptide having an amino acid sequence of formula [I] in which 113th Tyr was replaced by Cys

TNF-115Q: Polypeptide having an amino acid sequence of formula [I] in which 115th Pro was replaced by Gln

TNF-115A: Polypeptide having an amino acid sequence of formula [I] in which 115th Pro was replaced by Ala

TNF-115F: Polypeptide having an amino acid sequence of formula [I] in which 115th Pro was replaced by Phe

TNF-115N: Polypeptide having an amino acid sequence of formula [I] in which 115th Pro was replaced by Asn

TNF-115Y: Polypeptide having an amino acid sequence of formula [I] in which 115th Pro was replaced by Tyr

TNF-115V: Polypeptide having an amino acid sequence of formula [I] in which 115th Pro was replaced by Val

TNF-115E: Polypeptide having an amino acid sequence of formula [I] in which 115th Pro was replaced by Glu

TNF-115M: Polypeptide having an amino acid sequence of formula [I] in which 115th Pro was replaced by Met

TNF-115I: Polypeptide having an amino acid sequence of formula [I] in which 115th Pro was replaced by Ile

TNF-115W: Polypeptide having an amino acid sequence of formula [I] in which 115th Pro was replaced by Trp

TNF-115K: Polypeptide having an amino acid sequence of formula [I] in which 115th Pro was replaced by Lys

TNF-118Q: Polypeptide having an amino acid sequence of formula [I] in which 118th Leu was replaced by Gln

TNF-132T: Polypeptide having an amino acid sequence of formula [I] in which 132nd Ala was replaced by Thr

TNF-145C: Polypeptide having an amino acid sequence of formula [I] in which 145th Ser was replaced by Cys

TNF-153L: Polypeptide having an amino acid sequence of formula [I] in which 153rd Ile was replaced by Leu

Referential Example 1

Construction of an Expression Plasmid for Producing Human TNF:-

The cloned cDNA encoding human TNF was isolated by digestion with restriction endonuclease PstI, from the recombinant plasmid pHTNF13 prepared according to the method described in European Patent Publication No. 155549.

The cloned cDNA was digested with restriction endonuclease EcoRI to split off part of the non-coding region downstream of the TNF coding region. The resulting DNA fragment (about 1.1 kbp) was inserted into a larger DNA fragment prepared from a plasmid pBR322 by digestion with restriction endonucleases PstI and EcoRI to construct a recombinant plasmid including TNF cDNA and a tetracycline-resistance gene, which was named pHT113.

The recombinant plasmid pHT113 was digested with restriction endonucleases AvaI and SalI to cut it into three fragments (about 0.8 kbp, 1.3 kbp and 2.6 kbp in size). The 1.3 kbp-DNA fragment including most of the coding region for the human TNF and part of tetracycline-resistance gene was isolated (to be referred to as AvaI-SalI fragment). The Aval-SalI fragment was ligated with the following chemically synthesized oligodeoxyribonucleotide adapter [f].

```
5'-CGATATGTCATCTTCTCGAACC-3'          [f]
  3'-TATACAGTAGAAGAGCTTGGGGCT-5'
```

The resulting DNA fragment is referred to as HTNF-adapter fragment.

Separately, a DNA fragment (35 bp) including part of the trp promoter region was cut out from a plasmid pDR720 [P-L Biochemicals; D. R. Russell, et al., Gene, 20, 231 (1983)] by digesting with restriction endonucleases EcoRI and HpaI. The nucleotide sequence of the isolated 35 bp-DNA fragment is as follows:

5'-AATTCCCCTGTTGACAATTAATCATCGAACTAGTT-3'
3'-GGGGACAACTGTTAATTAGTAGCTTGATCAA-5'

The 35 bp-DNA fragment was ligated with a chemically synthesized adapter represented by the following formula:

5'-AACTAGTACGCAAGTTCACGTAAAAAGGGTAAT-3' [g]
3'-TTGATCATGCGTTCAAGTGCATTTTTCCCATTAGC-5'

The resulting DNA fragment is referred to as trp promoter fragment.

A plasmid pBR322 was digested with restriction endonucleases EcoRI and SalI, and then the larger DNA fragment (about 3.7 kbp) was isolated.

An expression plasmid for producing human TNF consisting of 155 amino acids corresponding to the amino acid sequence from amino acid No. 79 to No. 233 in Table 8, was constructed by sequential ligation of these three DNA fragments, the HTNF-adapter fragment, the trp promoter fragment and the larger pBR322 fragment (about 3.7 kbp) as illustrated in Figure. 9.

The expression plasmid was named pHTR91.

Referential Example 2

Construction of an Expression Plasmid for Producing a Modified Human TNF Polypeptide (147):-

An expression plasmid (pHT147) for producing a modified human TNF polypeptide consisting of 147 amino acids corresponding to amino acid No. 84 to No. 233 shown in Table 8, which is referred to as polypeptide TNF(147), was constructed as illustrated in Fig. 10.

The recombinant plasmid pHTR91 prepared as mentioned in Referential Example 1 was digested with restriction endonucleases ClaI and BalI to cut it into four DNA fragments. Two smaller DNA fragments (about 113 bp and 0.6 kbp in size) were isolated by 5% polyacrylamide

gel electrophoresis. The smallest DNA fragment (113 bp) was further digested with restriction endonucleae DdeI to cut it into two fragment (47 bp and 66 bp), and the 47 bp-DNA fragment was isolated (to be referred to as 47 bp-fragment).

The 47 bp-fragment was ligated with two chemically synthesized adapters represented by the following formulae:

```
5'-CGATTATGAAGCCTGTAG-3'                    [h]
  3'-TAATACTTCGGACATCGGG-5'
```

and

```
5'-CCCATGTTGTAGCAAACCCTCAAGC-3'             [i]
   3'-TACAACATCGTTTGGGAGTTCGACT-5'
```

Furthermore, the resulting DNA fragment was ligated with the following chemically synthesized adapter [j].

```
5'-AACTAGTACGCAAGTTCACGTAAGGAGGTTAT-3'  [j]
3'-TTGATCATGCGTTCAAGTGCATTCCTCCAATAGC-5'
```

The ligated DNA fragment is referred to as 5'-DNA fragment.

Separately, a DNA fragment (about 380 bp in size) containing the trp promoter region was isolated from a plasmid pCT-1 [M. Ikehara et al., Proc. Natl. Acad. Sci. USA, 81, 5956 (1984)] by double digestion with restriction endonucleases HpaI and AatII.

The base sequence of the trp promoter region of the above 380 bp DNA fragment was reported by Bennett et al. [J. Mol. Biol., 121, 113 (1978)]. This DNA fragment was combined with the 5'-DNA fragment by using T4 DNA ligase.

The ligated DNA fragment is referred to as Promoter-5'-DNA fragment.

The DNA fragment (487 bp in size) containing a base sequence corresponding to the C-terminal region of human TNF polypeptide was cut out from the recombinant

plasmid pHTR91 prepared as mentioned in Referential Example 1, by double digestion with restriction endonucleases BalI and HindIII, and isolated.

This DNA fragment was ligated with the Promoter-5'-DNA framgent by T4 DNA ligase. The ligated DNA fragment is referred to as Promoter-TNF(147)-DNA fragment.

Separately, the plasmid vector pBRS6 as shown in Example 1-(1) was cleaved with restriction endonucleases AatII and HindIII into two fragments. The larger DNA fragment (about 3.6 kbp) was isolated, and ligated by using T4 DNA ligase with the Promoter-TNF(147)-DNA fragment previously prepared in order to construct an expression plasmid pTH147 for producing the polypeptide TNF(147).

Referential Example 3

Construction of an Expression Plasmid for Producing Human TNF Polypeptide Mutant TNF-67S:-

An expression plasmid (pHTP392) for producing a polypeptide consisting of 155 amino acid and having an amino acid sequence corresponding to the sequence from amino acid No. 1 to No. 155 in Table 1, in which a cysteine residue in the 67th position from the N-terminus was replaced by a serine residue, referred to as TNF-67S, was constructed as illustrated in Figure. 11.

The recombinant plasmid pHTR91 prepared as mentioned in Referential Example 1 was digested with restriction endonucleases AvaI and HindIII to isolate about 600 bp DNA fragment corresponding to the base sequence of the downstream region from base No. 250 in Table 8. The 600 bp-DNA fragment was further digested with restriction endonucleases AvaII and HgiAI to cleave it into three DNA fragments (about 162 bp, 41 bp and 375 bp in size), and the 162 bp-DNA fragment and 375 bp-DNA fragment were isolated by polyacrylamide gel electrophoresis.

These DNA fragments were ligated with the following two chemically synthesized oligodeoxyribunucleotide adapters, [k] and [l], by using T4 DNA ligase.

```
5'-GTCCTCTTCAAGGGCCAA-3'                [k]
   3'-GAGAAGTTCCCGGTTCCGA-5'
```

and

```
5'-GGCTCTCCCTCCACCCATGTGCT-3'           [l]
    3'-GAGGGAGGTGGGTAC-5'
```

Furthermore, the resulting DNA fragment was sequentially ligated with the following two chemically synthesized oligodeoxyribonucleotide adapters, [j] and [m].

```
5'-AACTAGTACGCAAGTTCACGTAAGGAGGTTAT-3'  [j]
3'-TTGATCATGCGTTCAAGTGCATTCCTCCAATAGC-5'
```

and

```
5'-CGATTATGTCATCTTCTCGAACC-3'           [m]
  3'-TAATACAGTAGAAGAGCTTGGGGCT-5'
```

The resulting DNA fragment is referred to as TNF(Ser67)-DNA fragment.

The DNA fragment (about 380 bp) containing the trp promoter region was isolated from a plasmid pCT-1 by double digestion with restriction endonucleases HpaI and AatII as shown in Referential Example 2.

This DNA fragment was combined with the TNF(Ser67)-DNA framgnet by using T4 DNA ligase. The ligated DNA fragment is referred to as Promoter-TNF(Ser67)-DNA fragment.

Separately, the plasmid vector pBRS6 prepared as mentioned in Example 1-(1) was cleaved with restriction endonucleases AatII and HindIII into two fragments. The larger DNA fragemnt (about 3.6 kbp) was isolated, and ligated by T4 DNA ligase with the Promoter-TNF(Ser67)-DNA fragment previously prepared in order to construct expression plasmid pHTP392 for producing the TNF-67S.

In accordance with Example 1-(2), the plasmid was introduced into Escherichia coli. The transformant was cultured to produce TNF-67S.

Referential Example 4

Expression plasmids for production of the following polypeptides were constructed in accordance with Example 1-(1) and (2). The polypeptides were produced in Escherichia coli transformed with the expression plasmids.

TNF-70Y: Polypeptide having an amino acid sequence of formula [I] in which 70th Thr was replaced by Tyr

TNF-99S: Polypeptide having an amino acid sequence of formula [I] in which 99th Cys was replaced by Ser

Referential Example 5

In accordance with Example 1-(1) and (2), expression plasmids for production of the following polypeptides were constructed. Attempts were made to produce the polypeptides in Escherichia coli transformed with these expression plasmids. These polypeptides could not be extracted as soluble polypeptides or could be extracted only in small amounts.

TNF-12T: Polypeptide having an amino acid sequence of formula [I] in which 12th Ala was replaced by Thr

TNF-13Y: Polypeptide having an amino acid sequence of formula [I] in which 13th His was replaced by Tyr

TNF-14A: Polypeptide having an amino acid sequence of formula [I] in which 14th Val was replaced by Ala

TNF-17T: Polypeptide having an amino acid sequence of formula [I] in which 17th Asn was replaced by Thr

TNF-24F: Polypeptide having an amino acid sequence of formula [I] in which 24th Leu was replaced by Phe

TNF-26R: Polypeptide having an amino acid sequence of formula [I] in which 26th Trp was replaced by Arg

TNF-35P: Polypeptide having an amino acid sequence of formula [I] in which 35th Leu was replaced by Pro

TNF-44D: Polypeptide having an amino acid sequence of formula [I] in which 44th Asn was replaced by Asp

TNF-45P: Polypeptide having an amino acid sequence of formula [I] in which 45th Gln was replaced by Pro

TNF-50P: Polypeptide having an amino acid sequence of formula [I] in which 50th Ser was replaced by Pro

TNF-54C: Polypeptide having an amino acid sequence of formula [I] in which 54th Tyr was replaced by Cys

TNF-54H: Polypeptide having an amino acid sequence of formula [I] in which 54th Tyr was replaced by His

TNF-58P: Polypeptide having an amino acid sequence of formula [I] in which 58th Ser was replaced by Pro

TNF-59L: Polypeptide having an amino acid sequence of formula [I] in which 59th Gln was replaced by Leu

TNF-60D: Polypeptide having an amino acid sequence of formula [I] in which 60th Val was replaced by Asp

TNF-60G: Polypeptide having an amino acid sequence of formula [I] in which 60th VAl was replaced by Gly

TNF-62S: Polypeptide having an amino acid sequence of formula [I] in which 62nd Phe was replaced by Ser

TNF-93P: Polypeptide having an amino acid sequence of formula [I] in which 93rd Ser was replaced by Pro

TNF-121G: Polypeptide having an amino acid sequence of formula [I] in which 121st Val was replaced by Gly

TNF-124Q: Polypeptide having an amino acid sequence of formula [I] in which 124th Leu was replaced by Gln

TNF-128A: Polypeptide having an amino acid sequence of formula [I] in which 128th Asp was replaced by Ala

TNF-128N: Polypeptide having an amino acid sequence of formula [I] in which 128th Asp was replaced by Asn

TNF-135D: Polypeptide having an amino acid sequence of formula [I] in which 135th Asn was replaced by Asp

TNF-138Y: Polypeptide having an amino acid sequence of formula [I] in which 138th Asp was replaced by Tyr

TNF-148D: Polypeptide having an amino acid sequence of formula [I] in which 148th Val was replaced by Asp

TNF-148G: Polypeptide having an amino acid sequence of formula [I] in which 148th Val was replaced by Gly

TNF-150L: Polypeptide having an amino acid sequence of formula [I] in which 150th Phe was replaced by Leu

TNF-151E: Polypeptide having an amino acid sequence of formula [I] in which 151th Gly was replaced by Glu

Table 8

```
                                                GACCCACGG

-30         -20         -10         -1
 CTCCACCCTCTCTCCCCTGGAAAGGACACC
 1          10          20          30
 ATGAGCACTGAAAGCATGATCCGGGACGTG
 MetSerThrGluSerMetIleArgAspVal
                                                        10
           40          50          60
 GAGCTGGCCGAGGAGGCGCTCCCCAAGAAG
 GluLeuAlaGluGluAlaLeuProLysLys
                                                        20
            70          80          90
 ACAGGGGGGCCCCAGGGCTCCAGGCGGTGC
 ThrGlyGlyProGlnGlySerArgArgCys
                                                        30
           100         110         120
 TTGTTCCTCAGCCTCTTCTCCTTCCTGATC
 LeuPheLeuSerLeuPheSerPheLeuIle
                                                        40
           130         140         150
 GTGGCAGGCGCCACCACGCTCTTCTGCCTG
 ValAlaGlyAlaThrThrLeuPheCysLeu
                                                        50
           160         170         180
 CTGCACTTTGGAGTGATCGGCCCCCAGAGG
 LeuHisPheGlyValIleGlyProGlnArg
                                                        60
           190         200         210
 GAAGAGTTCCCCAGGGACCTCTCTCTAATC
 GluGluPheProArgAspLeuSerLeuIle
                                                        70
           220         230         240
 AGCCCTCTGGCCCAGGCAGTCAGATCATCT
 SerProLeuAlaGlnAlaValArgSerSer
                                                        80
```

- to be continued -

Table 8 (continued)

```
        250         260         270
TCTCGAACCCCGAGTGACAAGCCTGTAGCC
SerArgThrProSerAspLysProValAla
                                 90
        280         290         300
CATGTTGTAGCAAACCCTCAAGCTGAGGGG
HisValValAlaAsnProGlnAlaGluGly
                                100
        310         320         330
CAGCTCCAGTGGCTGAACCGCCGGGCCAAT
GlnLeuGlnTrpLeuAsnArgArgAlaAsn
                                110
        340         350         360
GCCCTCCTGGCCAATGGCGTGGAGCTGAGA
AlaLeuLeuAlaAsnGlyValGluLeuArg
                                120
        370         380         390
GATAACCAGCTGGTGGTGCCATCAGAGGGC
AspAsnGlnLeuValValProSerGluGly
                                130
        400         410         420
CTGTACCTCATCTACTCCCAGGTCCTCTTC
LeuTryLeuIleTyrSerGlnValLeuPhe
                                140
        430         440         450
AAGGGCCAAGGCTGCCCCTCCACCCATGTG
LysGlyGlnGlyCysProSerThrHisVal
                                150
        460         470         480
CTCCTCACCCACACCATCAGCCGCATCGCC
LeuLeuThrHisThrIleSerArgIleAla
                                160
        490         500         510
GTCTCCTACCAGACCAAGGTCAACCTCCTC
ValSerTyrGlnThrLysValAsnLeuLeu
                                170
```

- to be continued -

Table 8 (continued)

```
         520         530         540
TCTGCCATCAAGAGCCCCTGCCAGAGGGAG
SerAlaIleLysSerProCysGlnArgGlu
                                 180
         550         560         570
ACCCCAGAGGGGGCTGAGGCCAAGCCCTGG
ThrProGluGlyAlaGluAlaLysProTrp
                                 190
         580         590         600
TATGAGCCCATCTATCTGGGAGGGGTCTTC
TyrGluProIleTyrLeuGlyGlyValPhe
                                 200
         610         620         630
CAGCTGGAGAAGGGTGACCGACTCAGCGCT
GlnLeuGluLysGlyAspArgLeuSerAla
                                 210
         640         650         660
GAGATCAATCGGCCCGACTATCTCGACTTT
GluIleAsnArgProAspTyrLeuAspPhe
                                 220
         670         680         690
GCCGAGTCTGGGCAGGTCTACTTTGGGATC
AlaGluSerGlyGlnValTyrPheGlyIle
                                 230
         700         710         720
ATTGCCCTGTGAGGAGGACGAACATCCAAC
IleAlaLeu
         730         740
CTTCCCAAACGCCTCCCCTGC
```

Table 9

```
1          10          20          30
TCATCTTCTCGAACCCCGAGTGACAAGCCT
SerSerSerArgThrProSerAspLysPro
 1                              10
          40          50          60
GTAGCCCATGTTGTAGCAAACCCTCAAGCT
ValAlaHisValValAlaAsnProGlnAla
                               20
          70          80          90
GAGGGGCAGCTCCAGTGGCTGAACCGCCGG
GluGlyGlnLeuGlnTrpLeuAsnArgArg
                               30
         100         110         120
GCCTATGCCCTCCTGGCCAATGGCGTGGAG
AlaTyrAlaLeuLeuAlaAsnGlyValGlu
                               40
         130         140         150
CTGAGAGATAACCAGCTGGTGGTGCCATCA
LeuArgAspAsnGlnLeuValValProSer
                               50
         160         170         180
GAGGGCCTGTACCTCATCTACTCCCAGGTC
GluGlyLeuTryLeuIleTyrSerGlnVal
                               60
         190         200         210
CTCTTCAAGGGCCAAGGCTGCCCCTCCACC
LeuPheLysGlyGlnGlyCysProSerThr
                               70
         220         230         240
CATGTGCTCCTCACCCACACCATCAGCCGC
HisValLeuLeuThrHisThrIleSerArg
                               80
         250         260         270
ATCGCCGTCTCCTACCAGACCAAGGTCAAC
IleAlaValSerTyrGlnThrLysValAsn
                               90
```

- to be continued -

Table 9 (continued)

```
        280         290         300
CTCCTCTCTGCCATCAAGAGCCCCTGCCAG
LeuLeuSerAlaIleLysSerProCysGln
                                100
        310         320         330
AGGGAGACCCCAGAGGGGGCTGAGGCCAAG
ArgGluThrProGluGlyAlaGluAlaLys
                                110
        340         350         360
CCCTGGTATGAGCCCATCTATCTGGGAGGG
ProTrpTyrGluProIleTyrLeuGlyGly
                                120
        370         380         390
GTCTTCCAGCTGGAGAAGGGTGACCGACTC
ValPheGlnLeuGluLysGlyAspArgLeu
                                130
        400         410         420
AGCGCTGAGATCAATCGGCCCGACTATCTC
SerAlaGluIleAsnArgProAspTyrLeu
                                140
        430         440         450
GACTTTGCCGAGTCTGGGCAGGTCTACTTT
AspPheAlaGluSerGlyGlnValTyrPhe
                                150
        460
GGGATCATTGCCCTGTGA
GlyIleIleAlaLeu***
            155
```

Table 10

```
1          10          20          30
TCATCTTCTCGAACCCCGAGTGACAAGCCT
SerSerSerArgThrProSerAspLysPro
 1                              10
           40          50          60
GTAGCCCATGTTGTAGCAAACCCTCAAGCT
ValAlaHisValValAlaAsnProGlnAla
                                  20
           70          80          90
GAGGGGCAGCTCCAGTGGCTGAACCGCCGG
GluGlyGlnLeuGlnTrpLeuAsnArgArg
                                  30
          100         110         120
GCCTATGCCCTCCTGGCCAATGGCGTGGAG
AlaTyrAlaLeuLeuAlaAsnGlyValGlu
                                  40
          130         140         150
CTGAGAGATAACCAGCTGGTGGTGCCATCA
LeuArgAspAsnGlnLeuValValProSer
                                  50
          160         170         180
GAGGGCCTGTACCTCATCTACTCCCAGGTC
GluGlyLeuTryLeuIleTyrSerGlnVal
                                  60
          190         200         210
CTCTTCAAGGGCCAAGGCTGCCCCTCCACC
LeuPheLysGlyGlnGlyCysProSerThr
                                  70
          220         230         240
CATGTGCTCCTCACCCACACCATCAGCCGC
HisValLeuLeuThrHisThrIleSerArg
                                  80
          250         260         270
ATCGCCGTCTCCTACCAGACCAAGGTCAAC
IleAlaValSerTyrGlnThrLysValAsn
                                  90
```

- to be continued -

Table 10 (continued)

```
        280         290         300
CTCCTCTCTGCCATCAAGAGCCCCTGCCAG
LeuLeuSerAlaIleLysSerProCysGln
                                100
        310         320         330
AGGGAGACCCCAGAGGGGGCTGAGGCCAAG
ArgGluThrProGluGlyAlaGluAlaLys
                                110
        340         350         360
CCCTGGTATGAGCTCATCTATCTGGGAGGG
ProTrpTyrGluLeuIleTyrLeuGlyGly
                                120
        370         380         390
GTCTTCCAGCTGGAGAAGGGTGACCGACTC
ValPheGlnLeuGluLysGlyAspArgLeu
                                130
        400         410         420
AGCGCTGAGATCAATCGGCCCGACTATCTC
SerAlaGluIleAsnArgProAspTyrLeu
                                140
        430         440         450
GACTTTGCCGAGTCTGGGCAGGTCTACTTT
AspPheAlaGluSerGlyGlnValTyrPhe
                                150
        460
GGGATCATTGCCCTGTGA
GlyIleIleAlaLeu***
            155
```

What is claimed is:

1. A polypeptide having an amino acid sequence represented by formula [I] below in which at least one of the 16th, 31st to 34th, 36th, 48th, 73rd, 82nd, 85th, 89th, 94th, 97th, 98th, 103rd, 113th, 115th, 117th, 118th, 131st, 132nd, 141st to 146th, and 153rd amino acid residues is replaced by another amino acid residue, with the proviso that when the 115th amino acid residue is replaced by another amino acid residue, the 67th amino acid residue and/or the 99th amino acid residue may be replaced by another amino acid residue; or a polypeptide resulting from deletion of one or at most 8 successive amino acid residues from the N-terminus of said polypeptide:

Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro
Val Ala His Val Val Ala Asn Pro Gln Ala
Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
Ala Asn Ala Leu Leu Ala Asn Gly Val Glu
Leu Arg Asp Asn Gln Leu Val Val Pro Ser
Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val
Leu Phe Lys Gly Gln Gly Cys Pro Ser Thr
His Val Leu Leu Thr His Thr Ile Ser Arg
Ile Ala Val Ser Tyr Gln Thr Lys Val Asn
Leu Leu Ser Ala Ile Lys Ser Pro Cys Gln
Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys
Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly
Val Phe Gln Leu Glu Lys Gly Asp Arg Leu
Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu
Asp Phe Ala Glu Ser Gly Gln Val Tyr Phe
Gly Ile Ile Ala Leu ... [I]

2. A polypeptide according to claim 1 wherein

(A) at least one of the following replacements of amino acids in the amino acid sequence of formula [I] is effected:

16th Ala by Val,
31st Ala by Thr,
32nd Asn by Ala, Cys, Asp, His, Ile, Arg, Ser, Thr, Val or Tyr

34th Leu by Ile,
36th Ala by Val,
48th Val by Met,
73rd Leu by Pro,
82nd Ala by Asp,
85th Tyr by His,
89th Val by Ile,
94th Ala by Thr,
97th Ser by Asn,
98th Pro by His or Leu,
103rd Thr by Pro,
113th Tyr by Cys,
115th Pro by Leu, His, Gln, Ser, Ala, Phe, Asn, Gly, Tyr, Val, Glu, Met, Ile, Asp, Trp, Lys, Arg, or Thr,
117th Tyr by His,
118th Leu by Gln,
131st Ser by Ile,
132nd Ala by Thr,
141st Asp by Tyr,
143rd Ala by Val,
144th Glu by Lys,
145th Ser by Cys,
146th Gly by Glu, and
153rd Ile by Leu;

(B) 67th Cys and/or 99th Cys are replaced by Ser and 115th Pro is replaced by an amino acid other than Pro in an amino acid sequence represented by formula [I]; or

(C) the polypeptide (A) or (B) in which one or at most 8 successive amino acid residues from the N-terminus is deleted;

3. A polypeptide according to claim 1 wherein in the amino acid sequence represented by formula [I], at least one of the following replacements of amino acids is effected:

16th Ala by Val,
36th Ala by Val,
73rd Leu by Pro,
98th Pro by His or Leu,
103rd Thr by Pro,
115th Pro by His or Gln,
131st Ser by Ile, and
143rd Ala by Val.

4. A polypeptide according to claim 1 wherein in the amino acid sequence of formula [I], at least one of the following replacements of amino acids is effected:

31st Ala by Thr,
32nd Asn by Ala, Cys, Asp, His, Ile, Arg, Ser, Thr, Val or Tyr,
115th Pro by Ser, Ala, Phe, Asn, Thr, Gly, Tyr, Val, Glu, Met, Ile, Asp, Trp, Leu or Lys, and
117th Tyr by His.

5. A polypeptide according to claim 1 wherein in the amino acid sequence of formula [I], at least one of the following replacements of amino acids is effected:

32nd Asn by Tyr, His, Asp or Ser,
115th Pro by Leu, Ser, Asp or Gly, and
117th Tyr by His.

6. A polypeptide according to claim 1 wherein in the amino acid sequence of formula [I], 32nd Asn or 115th Pro, or both, are replaced by other amino acids.

7. A polypeptide according to claim 1 wherein in the amino acid sequence of formula [I], 32nd Asn is replaced by Tyr.

8. A polypeptide of claim 1 wherein in the amino acid sequence of formula [I], 115th Pro is replaced by Leu.

9. A polypeptide of any one of claims 1 to 8 wherein Met is joined to the N-terminus of the amino acid sequence.

10. A DNA having a base sequence encoding a polypeptide according to any one of claims 1 to 9.

11. A DNA having a base sequence represented by the following formula [A] in which 32nd codon AAT and/or 115th codon CCC are replaced by codons encoding other amino acids:

(5') TCA TCT TCT CGA ACC CCG AGT GAC AAG
CCT GTA GCC CAT GTT GTA GCA AAC CCT CAA
GCT GAG GGG CAG CTC CAG TGG CTG AAC CGC
CGG GCC AAT GCC CTC CTG GCC AAT GGC GTG
GAG CTG AGA GAT AAC CAG CTG GTG GTG CCA
TCA GAG GGC CTG TAC CTC ATC TAC TCC CAG
GTC CTC TTC AAG GGC CAA GGC TGC CCC TCC
ACC CAT GTG CTC CTC ACC CAC ACC ATC AGC
CGC ATC GCC GTC TCC TAC CAG ACC AAG GTC
AAC CTC CTC TCT GCC ATC AAG AGC CCC TGC
CAG AGG GAG ACC CCA GAG GGG GCT GAG GCC
AAG CCC TGG TAT GAG CCC ATC TAT CTG GGA
GGG GTC TTC CAG CTG GAG AAG GGT GAC CGA
CTC AGC GCT GAG ATC AAT CGG CCC GAC TAT
CTC GAC TTT GCC GAG TCT GGG CAG GTC TAC
TTT GGG ATC ATT GCC CTG-(3') ... [A]

12. A DNA according to claim 11 wherein in the base sequence of formula [A], 32nd codon AAT is replaced by a codon encoding another amino acid.

13. A DNA according to claim 11 wherein in the base sequence of formula [A], 115th codon CCC is replaced by a codon encoding another amino acid.

14. A DNA according to claim 12 wherein the codon encoding the other amino acid is TAT encoding Tyr.

15. A DNA according to claim 13 wherein the codon encoding the other amino acid is CTC encoding Leu.

16. A DNA according to any one of claims 10 to 15 wherein a translation initiation codon is joined to the 5'-terminus of the base sequence, and/or a termination codon is joined to the 3'-terminus of the base sequence.

17. A vector having inserted thereinto a DNA according to any one of claims 10 to 16.

18. A vector resulting from insertion of a DNA according to any one of claims 10 to 16 into an expression vector.

19. A host transformed with the vector of claim 17 or 18.

20. The host according to claim 19 which is a microorganism.

21. The host according to 19 or 20 which is Escherichia coli.

22. A method of producing a polypeptide according to any one of claims 1 to 9 which comprises culturing the host of any one of claims 19 to 20, and isolating the resulting polypeptide from the culture.

23. A pharmaceutical composition comprising a polypeptide according to any one of claims 1 to 9 or its salt.

FIG. 1

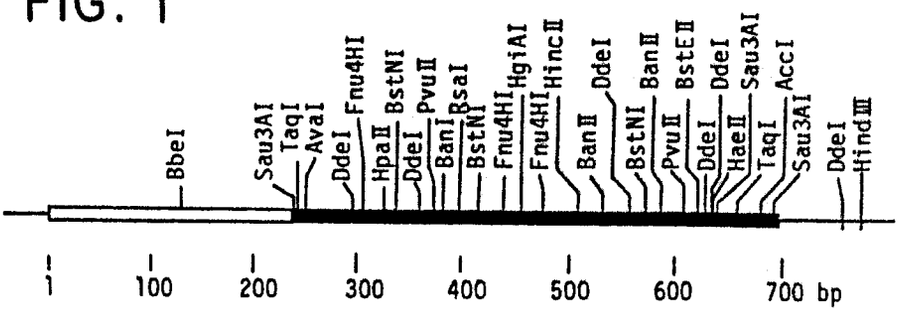
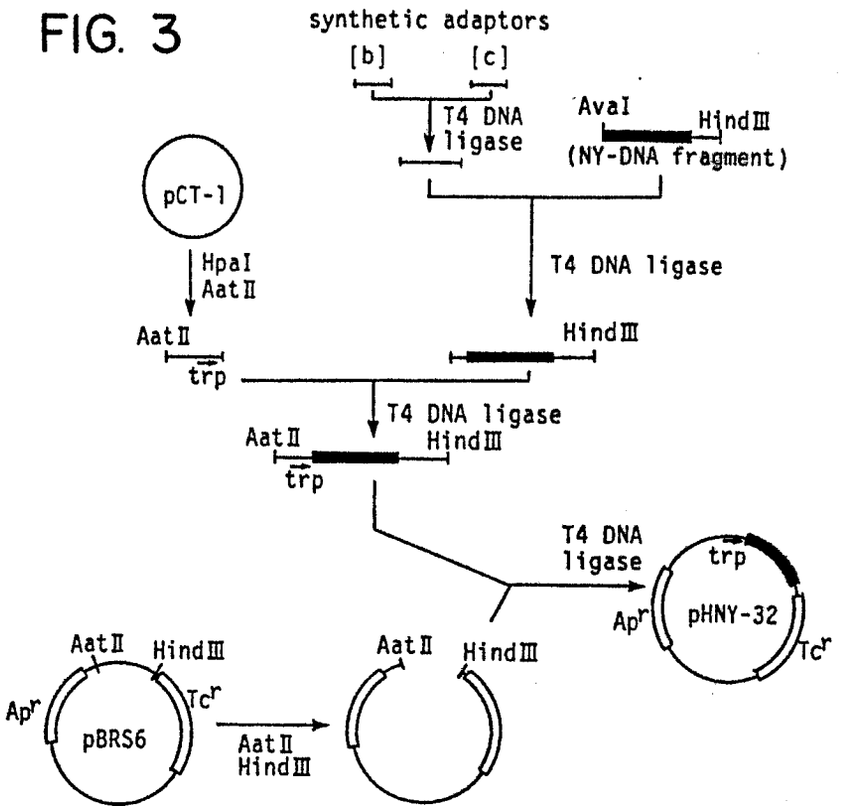

BbeI
Sau3AI
TaqI
AvaI
DdeI
Fnu4HI
HpaII
BstNI
DdeI
PvuII
BanI
BsaI
BstNI
Fnu4HI
HgiAI
Fnu4HI
HincII
BanII
DdeI
BstNI
BanII
PvuII
BstEII
DdeI
DdeI
HaeII
Sau3AI
TaqI
AccI
Sau3AI
DdeI
HindIII
1
100
200
300
400
500
600
700 bp

FIG. 3


synthetic adaptors
[b]
[c]
T4 DNA ligase
AvaI
HindIII
(NY-DNA fragment)
pCT-1
HpaI
AatII
T4 DNA ligase
AatII
HindIII
trp
T4 DNA ligase
AatII
HindIII
trp
T4 DNA ligase
trp
Ap^r
pHNY-32
Tc^r
AatII
HindIII
Ap^r
pBRS6
Tc^r
AatII
HindIII
AatII
HindIII

0251037

FIG. 2

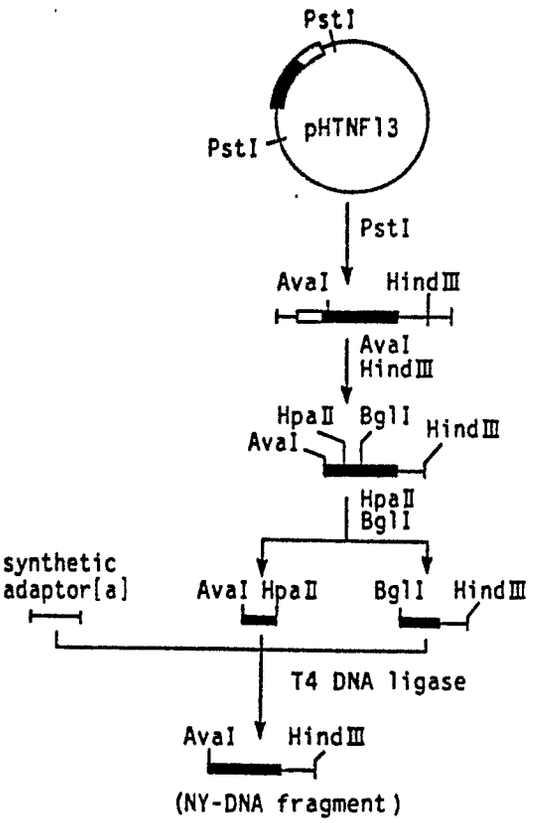
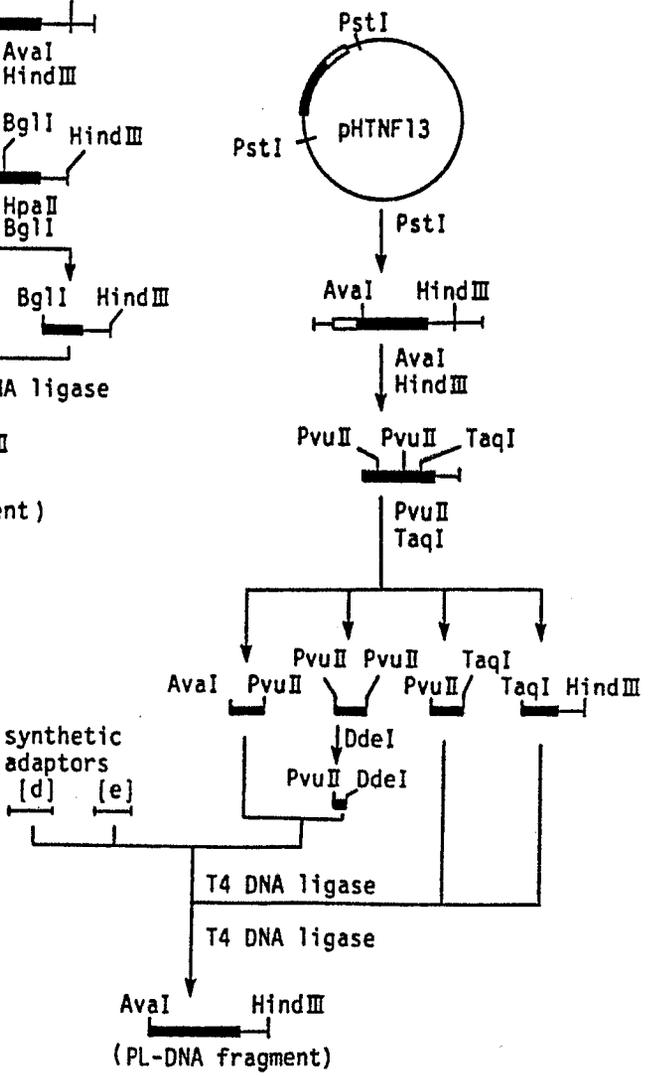


FIG. 4



0251037

FIG. 5

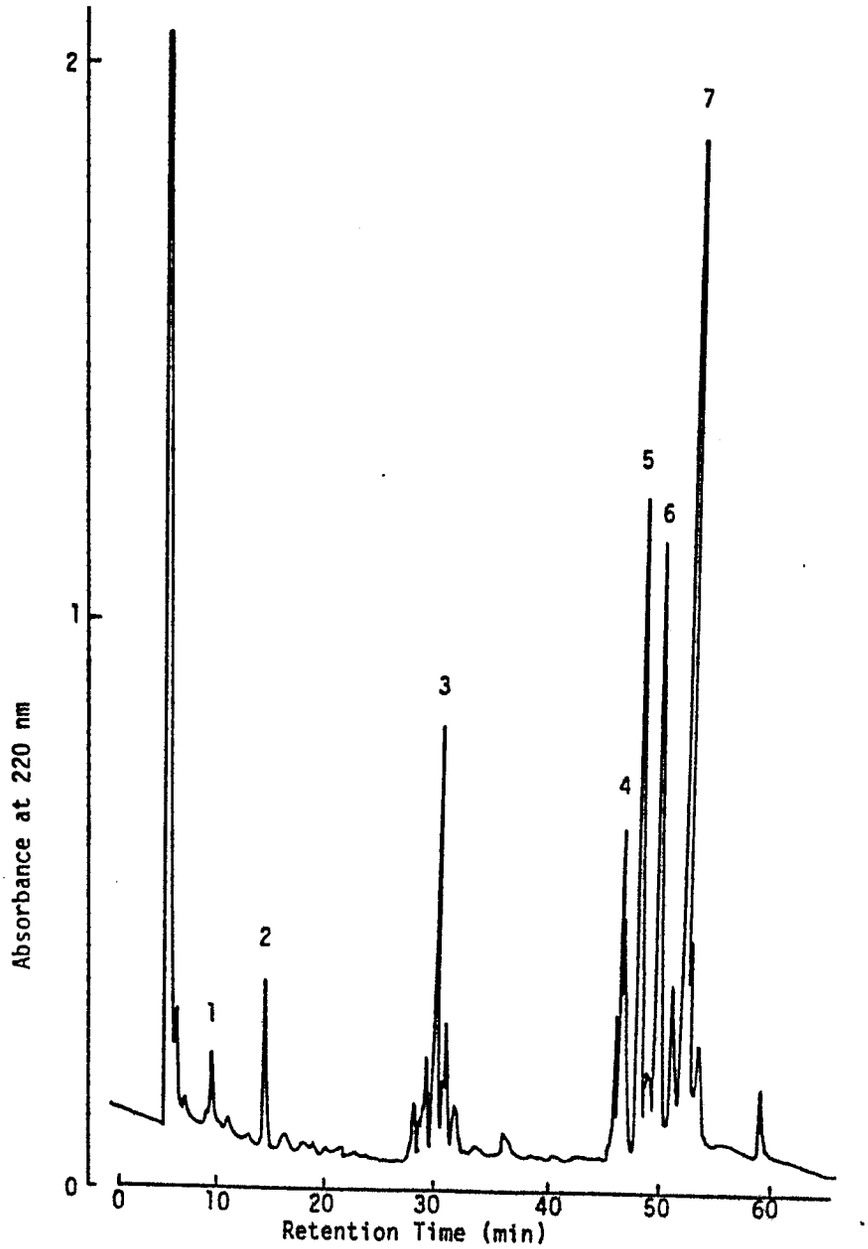


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FIG. 6

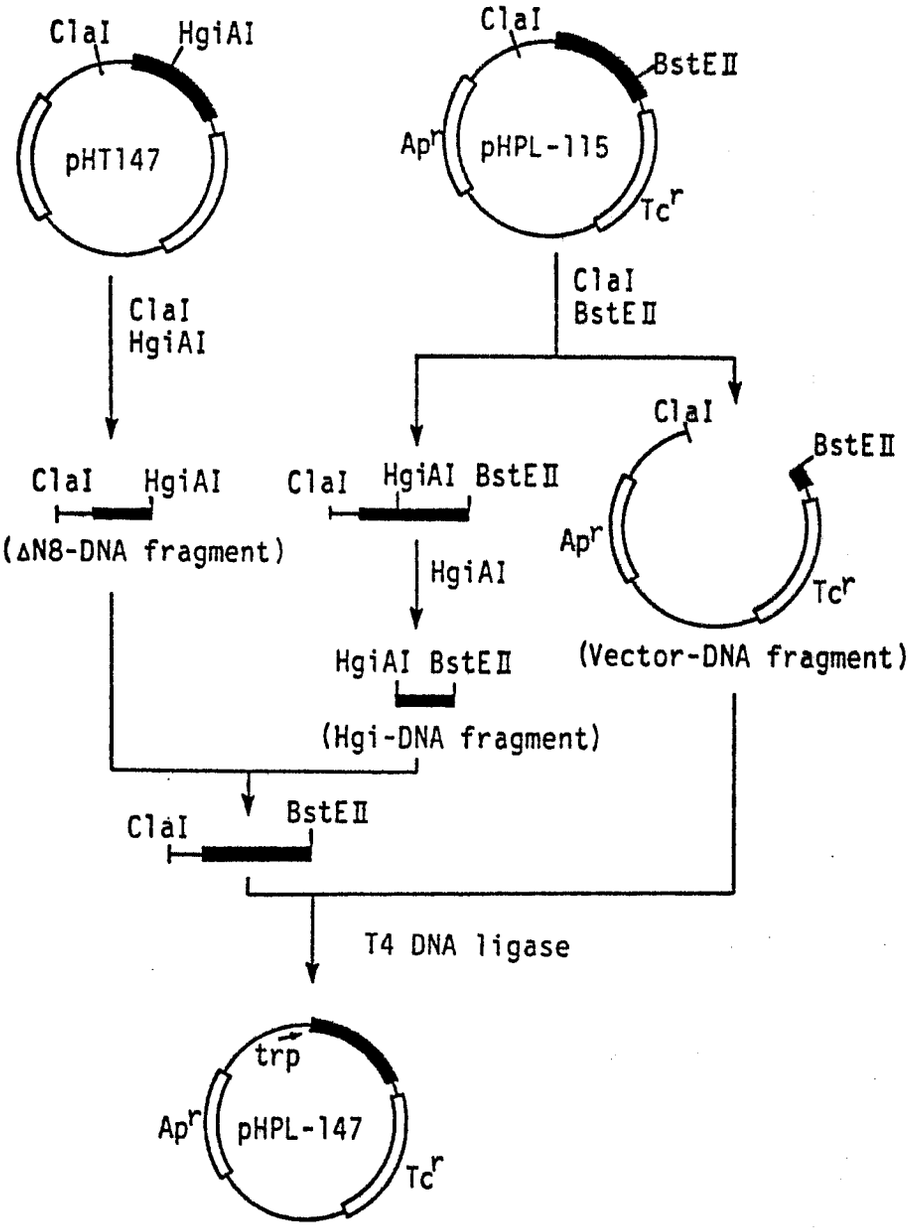
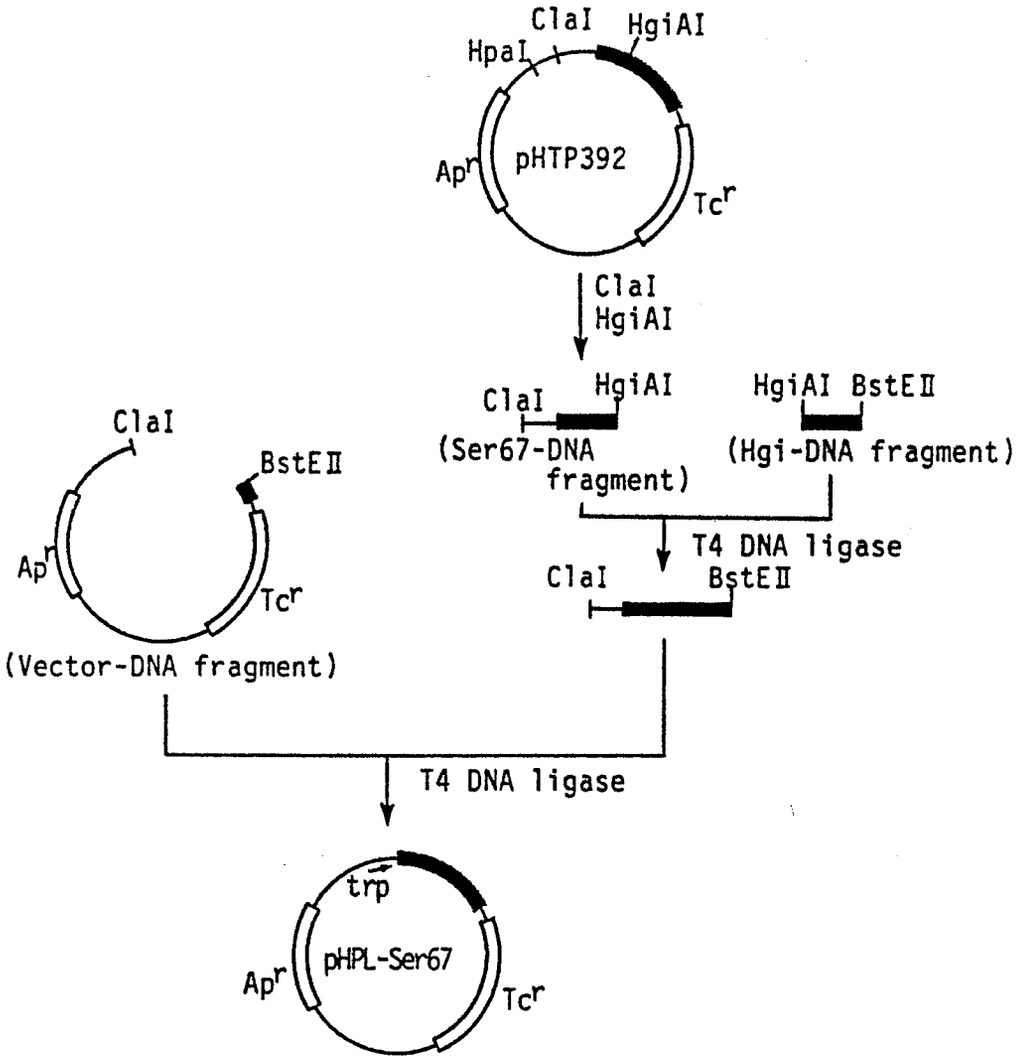


FIG. 7


ClaI
HgiAI
HpaI
pHTP392
Ap^r
Tc^r
ClaI
HgiAI
ClaI
HgiAI
HgiAI
BstEII
(Ser67-DNA fragment)
(Hgi-DNA fragment)
T4 DNA ligase
ClaI
BstEII
ClaI
BstEII
Ap^r
Tc^r
(Vector-DNA fragment)
T4 DNA ligase
trp
pHPL-Ser67
Ap^r
Tc^r

0251037

FIG. 8

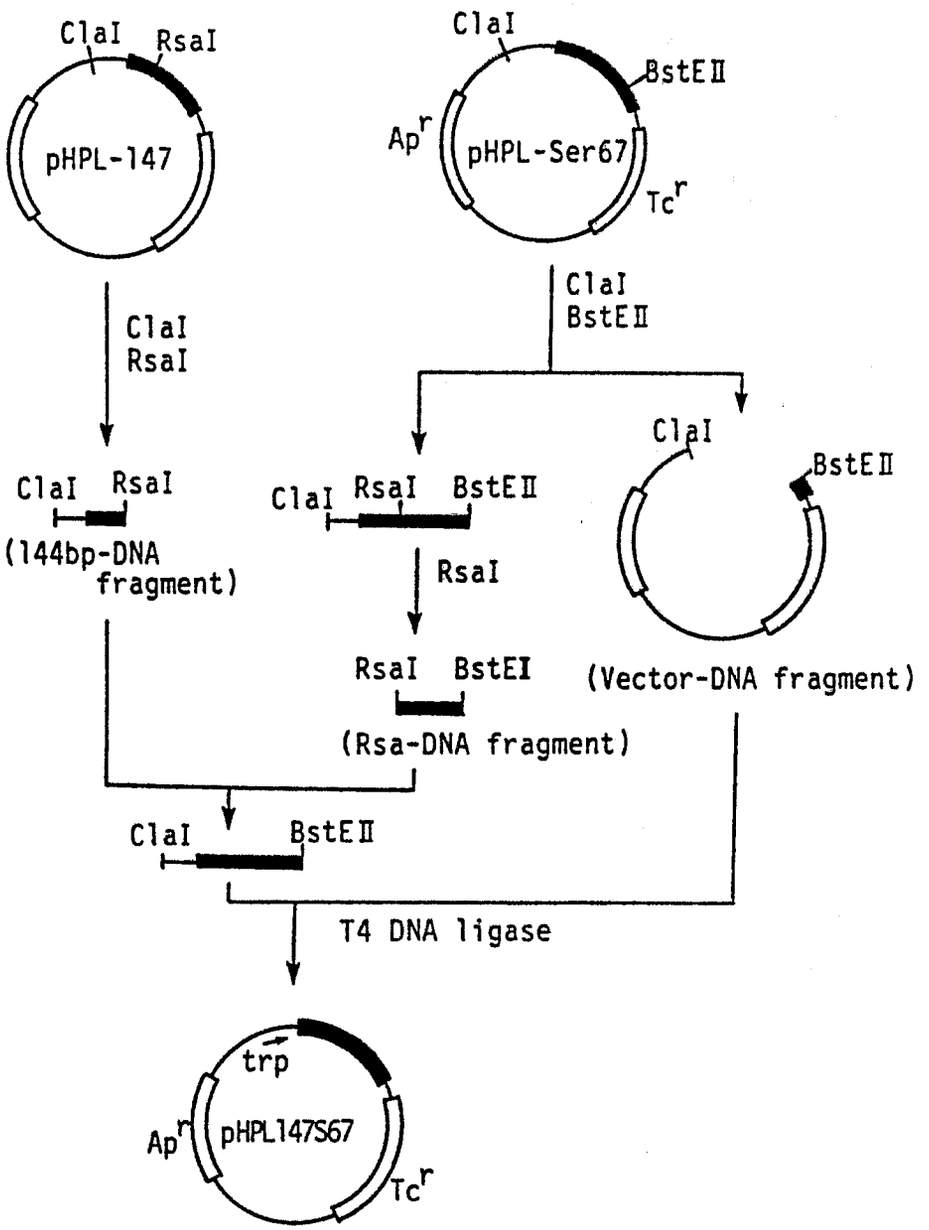
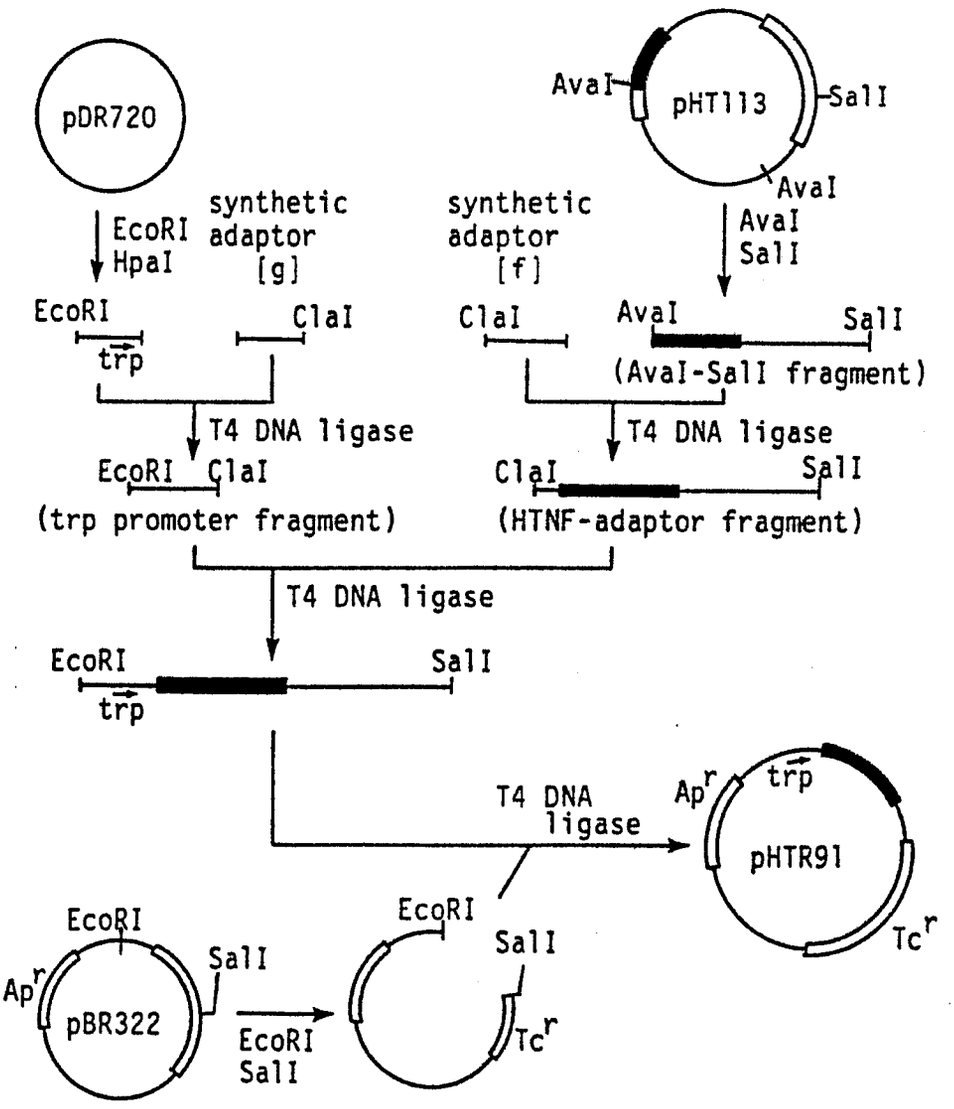


FIG. 9


pDR720
EcoRI
HpaI
synthetic
adaptor
[g]
EcoRI
trp
ClaI
T4 DNA ligase
EcoRI
ClaI
(trp promoter fragment)
AvaI
pHT113
SalI
AvaI
synthetic
adaptor
[f]
AvaI
SalI
ClaI
AvaI
SalI
(AvaI-SalI fragment)
T4 DNA ligase
ClaI
SalI
(HTNF-adaptor fragment)
T4 DNA ligase
EcoRI
trp
SalI
T4 DNA
ligase
Ap^r
trp
pHTR91
Tc^r
EcoRI
SalI
Ap^r
pBR322
EcoRI
SalI
EcoRI
SalI
Tc^r

8/9

FIG. 10

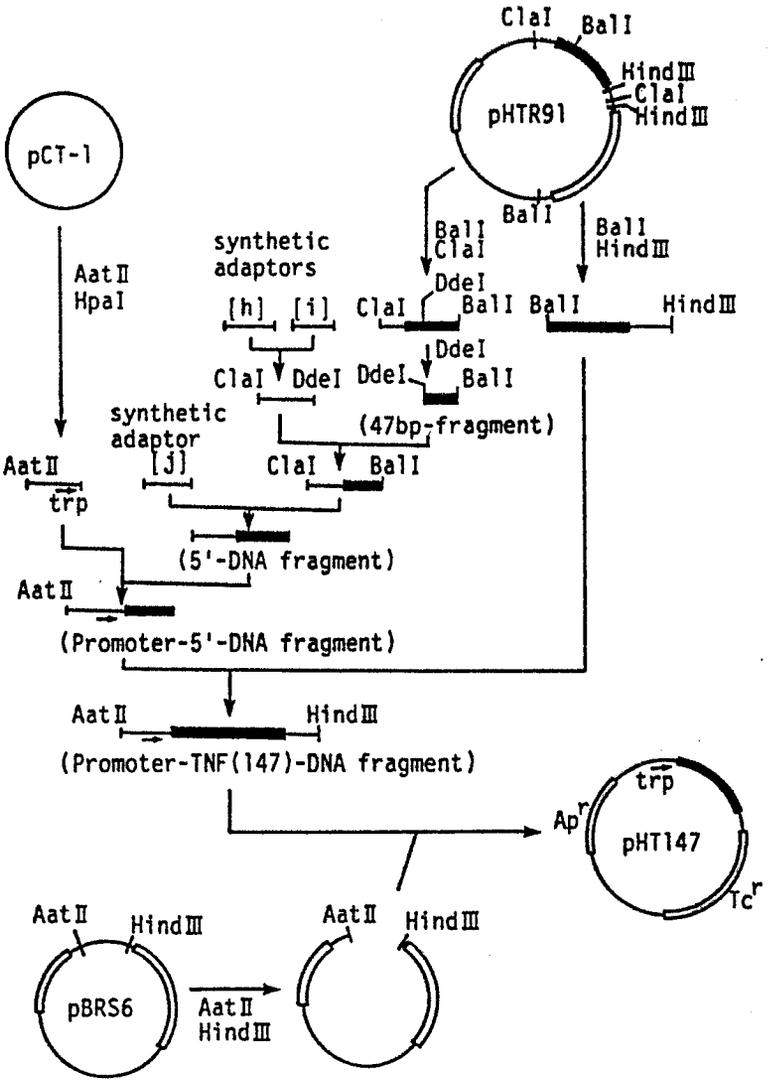
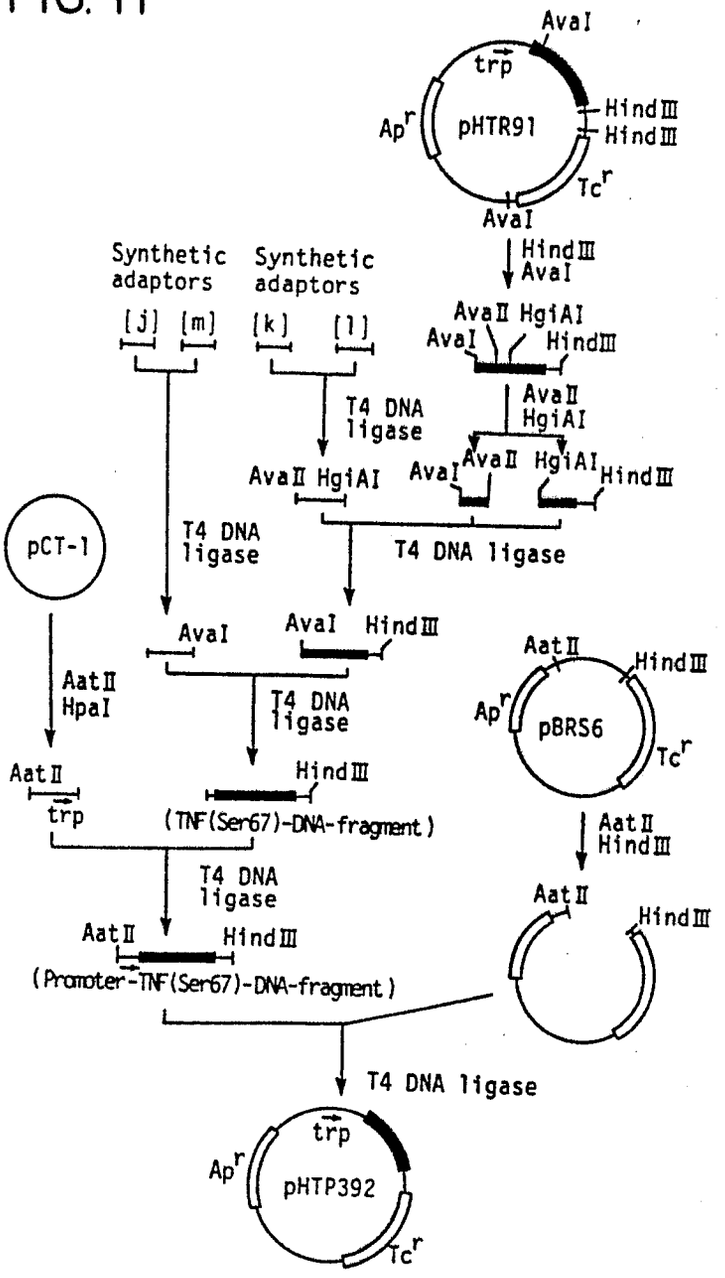

ClaI
BalI
HindIII
ClaI
HindIII
pHTR91
BalI
pCT-1
BalI
ClaI
BalI
HindIII
synthetic
adaptors
AatII
HpaI
DdeI
[h]
[i]
ClaI
BalI
BalI
HindIII
DdeI
ClaI
DdeI
DdeI
BalI
synthetic
adaptor
(47bp-fragment)
AatII
[j]
ClaI
BalI
trp
(5'-DNA fragment)
AatII
(Promoter-5'-DNA fragment)
AatII
HindIII
(Promoter-TNF(147)-DNA fragment)
trp
Ap^r
pHT147
Tc^r
AatII
HindIII
AatII
HindIII
pBRS6
AatII
HindIII

FIG. 11


AvaI
trp
Ap^r
pHTR91
HindIII
HindIII
Tc^r
AvaI
HindIII
AvaI
Synthetic adaptors
[j] [m]
Synthetic adaptors
[k] [l]
AvaII HgiAI
AvaI
HindIII
T4 DNA ligase
AvaII HgiAI
AvaII HgiAI
AvaI
AvaII HgiAI
HindIII
pCT-1
T4 DNA ligase
T4 DNA ligase
AvaI
AvaI HindIII
AatII HpaI
T4 DNA ligase
AatII
HindIII
AatII
trp
(TNF(Ser67)-DNA-fragment)
T4 DNA ligase
AatII
HindIII
(Promoter-TNF(Ser67)-DNA-fragment)
AatII
HindIII
Ap^r
pBRS6
Tc^r
AatII HindIII
AatII
HindIII
T4 DNA ligase
trp
Ap^r
pHTP392
Tc^r